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Pathophysiology of primary aldosteronism: adrenal immunohistochemistry and molecular profiling studies

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Lucie Sophie Meyer

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First supervisor:	Prof. Dr. med. Martin Reincke
Second supervisor:	Dr. Tracy Ann Williams
Third supervisor:	Prof. Dr. William E. Rainey
Dean:	Prof. Dr. med. Thomas Gudermann
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Contents

List of Abbreviations

ACTH	adrenocorticotropic hormone
APA	aldosterone-producing adenoma
APDH	aldosterone-producing diffuse hyperplasia
APM	aldosterone-producing micronodule
APN	aldosterone-producing nodule
ARR	aldosterone-to-renin-ratio
AVS	adrenal venous sampling
CYP11B1	11ß-hydroxylase
CYP11B2	aldosterone synthase
CYP17A1	17-α-hydroxylase
FFPE	formalin-fixed paraffin-embedded
HE	haematoxylin and eosin
HISTALDO	Histopathology of Primary Aldosteronism
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MALDI-MSI	Matrix-Assisted Laser Desorption/Ionization - Mass Spectrometry Imaging
MAPM	multiple aldosterone-producing micronodules
MAPN	multiple aldosterone-producing nodules
NGS	next-generation-sequencing
PA	primary aldosteronism
PASO	Primary Aldosteronism Surgical Outcome
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis

List of Publications and Awards

Scientific Publications Summarized in This Thesis

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2. Na Sun, Lucie S Meyer, Annette Feuchtinger, Thomas Kunzke, Thomas Knösel, Martin Reincke, Axel Walch, Tracy Ann Williams. **Mass Spectrometry Imaging Establishes 2 Distinct Metabolic Phenotypes of Aldosterone-Producing Cell Clusters in Primary Aldosteronism**, *Hypertension, 2020;75:634-644.* DOI: 10.1161/HYPERTENSIONAHA.119.14041

3. Lucie S Meyer, Laura Handgriff, Jung Soo Lim, Aaron M Udager, Isabella-Sabrina Kinker, Roland Ladurner, Moritz Wildgruber, Thomas Knösel, Martin Bidlingmaier, William E. Rainey, Martin Reincke, Tracy Ann Williams. **Single-Center Prospective Cohort Study on the Histopathology, Genotype, and Postsurgical Outcomes of Patients With Primary Aldosteronism**, *Hypertension, 2021;78:738–746.* DOI: 10.1161/HYPERTENSIONAHA.121.17348

Further Publications Resulting During my Time as PhD Candidate

1. Lucie S Meyer, Siyuan Gong, Martin Reincke, Tracy Ann Williams. **Angiotensin II type 1 receptor autoantibodies in primary aldosteronism** (invited review), *Hormone and Metabolic Research, 2020;52:379-385.*
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3. Lucie S Meyer, Martin Reincke, Tracy Ann Williams. **Timeline of Advances in Genetics of Primary Aldosteronism** (book chapter). *In: Igaz P., Patócs A. (editors) Genetics of Endocrine Diseases and Syndromes, Experientia Supplementum, vol 111. Springer, Cham.*
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Awards and Grants

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- **DGE Travel Grant**
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  Germany, 2018

Confirmation of co-authors

Hereby I declare that all authors contributed to the publications I-III gave written informed consent to use these publications in the frame of my dissertation.

1 Introductory Summary

1.1 Primary Aldosteronism

1.1.1 Introduction to Primary Aldosteronism

Hypertension is a major health issue in society that affects globally 1 in 4 men and 1 in 5 women [1]. Previously thought to be a rare disease, primary aldosteronism (PA) is now widely considered the most common form of endocrine hypertension with a prevalence of 3–20% depending on the severity of hypertension [2–5]. PA affects the quality of life when left untreated [6] and carries an increased risk of metabolic syndrome and diabetes [7]. In addition, PA is associated with an increased risk of left ventricular hypertrophy and elevated risk of cardiovascular events such as stroke, atrial fibrillation or heart failure compared to patients with essential hypertension [7]. Thus, it is of high importance to identify patients with PA and initiate timely specific treatment.

The cause of PA is inappropriate aldosterone excess from one or both adrenal glands relative to suppressed renin activity [8]. Once considered a requisite feature of the disease, the additional presence of hypokalaemia is only found in 9–37% of patients with PA [3, 9]. In 1954, Jerome Conn described the index case with PA with an aldosterone-producing adenoma (APA) and thus this endocrine disorder was named Conn-syndrome [10]. However, with the development of imaging techniques such as computed tomography scanning and methods to sample adrenal venous blood for hormone measurements, the following decades demonstrated that PA is caused not just by unilateral APAs but also by bilateral forms (referred to as bilateral adrenal hyperplasia or idiopathic hyperaldosteronism) associated with multinodular hyperplasia [8, 11]. Less common are cases of unilateral adrenocortical hyperplasia and very rare are adrenocortical aldosterone-producing carcinomas. Unilateral forms of PA are treated surgically in contrast to the medical treatment of bilateral forms of the disease [8].

1.1.2 Diagnosis

Recommendations for the diagnosis and treatment of patients with PA have been defined by the Endocrine Society Guideline [8]. The Guideline is followed in major referral centres for PA, including Munich, although each centre uses local reference values for screening

and confirmatory testing and interpretation of adrenal venous sampling results [12]. Briefly, candidates for PA are first screened using the aldosterone-to-renin-ratio (ARR) (see Figure 1.1) [8]. Patients with an ARR above an upper reference limit further undergo at least one confirmatory test to confirm or reject the diagnosis of PA [8].

Next, computed tomography of the adrenals in patients with a confirmed diagnosis of PA is conducted to rule out rare but malignant adrenocortical carcinoma [8]. Subsequently, adrenal venous sampling (AVS) is required to differentiate surgically-treated unilateral from bilateral PA [8]. AVS is a minimally invasive method in which a catheter is inserted into both adrenal veins to collect blood to identify which adrenal (or adrenals) overproduce aldosterone. Patients diagnosed with unilateral PA undergo unilateral laparoscopic adrenalectomy, while patients with bilateral PA are treated with antihypertensive medication, predominantly with mineralocorticoid receptor antagonists [8].

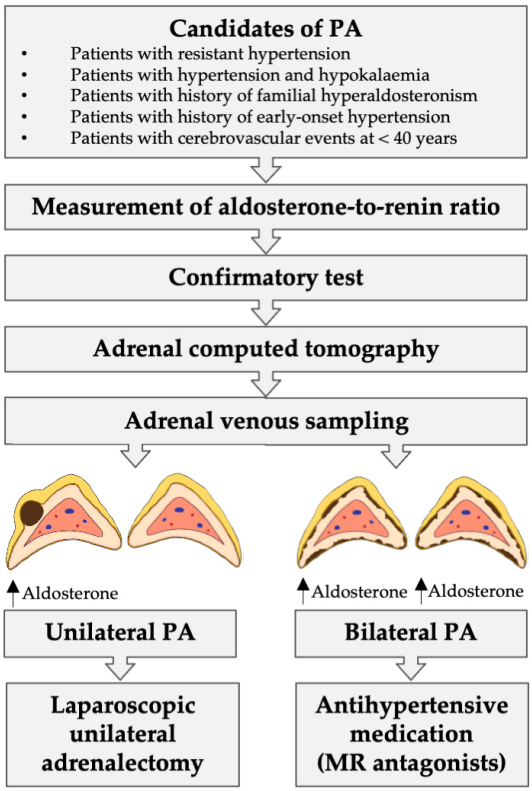

Figure 1.1: Simplified Flow-Chart of the Diagnostic Management of Primary Aldosteronism. MR, mineralocorticoid receptor; PA, primary aldosteronism.

1.1.3 Histopathology

The adrenal cortex is composed of three histologically distinguishable zones, each synthesizing different steroids from the common precursor cholesterol dependent on zone-specifically expressed enzymes [13, 14]. A capsule of connective tissue surrounds each adrenal gland [14]. Cells of the outermost zona glomerulosa (ZG) are small and densely packed in rosette structures [13, 14]. In mitochondria, aldosterone synthase, encoded by *CYP11B2* and confined to the ZG, completes the three successive final reactions of aldosterone synthesis [15]. Compared

to ZG cells, the cells of the middle zona fasciculata (ZF) are lipid-rich, contain more cytoplasm and are less densely packed [13]. These cells are structured in columns often close to blood vessels for rapid signaling responses upon adrenocorticotropic hormone (ACTH) stimulation [13]. In the ZF, the 11ß-hydroxylase and 17-α-hydroxylase, encoded by *CYP11B1* and *CYP17A1*, respectively, are responsible for cortisol synthesis [16]. The innermost zona reticularis (ZR) cells are slightly smaller and resemble ZF cells but with less lipid droplets and a higher number of lysosomes [13, 14]. ZR cells build structures resembling a net and synthesize androgens [13, 14].

With increasing age, starting at an age of about 12 years, the aldosterone-producing cells of the ZG become discontinuous and aldosterone-producing micronodules (APM, formerly called aldosterone-producing cell clusters APCCs) become more abundant [17, 18]. These APM accumulate over the next decades of life [17, 18]. They have been implicated in the pathophysiology of PA, which has been discussed extensively over the last few years [17, 18]. Over the last decades, the histopathological classification of adrenals from patients with PA has varied widely between different international centres. To simplify and unify the histopathological assessment and nomenclature of adrenal lesions from these patients, the Histopathology of Primary Aldosteronism (HISTALDO) consensus was established in 2021 from international experts in the field of PA [19]. These histopathological lesions comprise an APA, an aldosterone-producing nodule (APN), an aldosterone-producing micronodule (APM), multiple aldosterone-producing nodules (MAPN), multiple aldosterone-producing micronodules (MAPM), and aldosterone-producing diffuse hyperplasia (APDH) (see Table 1.1) [19]. The assessment of the adrenals is based on haematoxylin and eosin (HE) staining as well as immunohistochemical staining using the highly specific monoclonal antibody against the aldosterone synthase encoded by *CYP11B2* [20]. Adrenals from patients with PA can harbour different histopathological lesions of which some can also occur together in the same adrenal [21]. All these histopathologic phenotypes were observed in patients operated for unilateral PA. The predominant pharmacological treatment of patients with bilateral PA and thus scarce availability of resected adrenals from these patients means that the histopathological phenotype of patients with bilateral PA has been poorly examined to date. At the molecular level, APAs are the most intensely studied cause of PA and are associated with diverse clinical and histopathological genotype-phenotype correlations as described in

section 1.1.4.

Table 1.1: Nomenclature of histopathologic aldosterone-producing lesions in adrenals from patients with PA assessed according to the HISTALDO consensus [19].

Abbreviation	Name	Description
APA	aldosterone-producing adenoma	CYP11B2-positive, clearly circumscribed lesion of ≥10mm which can be distinguished from the adjacent adrenal cortex by HE staining
APN/MAPN	(multiple) aldosterone-producing nodules	one or multiple CYP11B2-positive lesions of <10mm which are morphologically visible by HE staining
APM/MAPM	(multiple) aldosterone-producing micronodules	one or multiple CYP11B2-positive lesions <10mm located beneath the capsule which are not morphologically visible by HE staining
APDH	aldosterone-producing diffuse hyperplasia	disrupted or continuous layer of the ZG which displays CYP11B2-positive cells in >50% of the ZG

1.1.4 Genetics

Somatic as well as germline variants have been identified as underlying causes of PA. The inherited forms of PA are rare with a prevalence of probably <1% and are mainly associated with early-onset forms of the disease [22]. Details of these familial forms, classified into familial hyperaldosteronism (FH) type I – IV based on the affected gene, are reviewed elsewhere [23, 24].

CYP11B2-guided sequencing approaches using next-generation sequencing (NGS) methods report a genetic variant in an aldosterone-driver gene in up to 96% of APAs or APNs [25]. The majority of genes identified so far which carry mutations associated with aldosterone overproduction are ion channels or ATPases. Those genes are: the G-protein activated inwardly rectifying potassium channel GIRK4 encoded by *KCNJ5* [26], the Na^+/K^+-ATPase encoded by *ATP1A1* [27, 28], the plasma membrane Ca^{2+}-transporting ATPase type 3 encoded by *ATP2B3* [28], the voltage-gated L-type calcium channel subunit α-1D encoded by *CACNA1D* [27, 29], the voltage-gated T-type calcium channel subunit α-1H encoded by

CACNA1H [30] and the voltage-gated chloride channel 2 encoded by *CLCN2* [31, 32]. Variants in these genes impact intracellular Ca^{2+} levels and thus activate Ca^{2+} signaling pathways that initiate aldosterone synthesis [14]. Besides these major aldosterone-driver genes, mutations associated with PA have also been identified in other genes such as *CTNNB1* which encodes ß-catenin [33, 34]. *CTNNB1* mutations appear to have role in tumor development rather than directly related to aldosterone secretion [33, 34].

The most frequently mutated gene in APAs in most studied populations is *KCNJ5*. The mean prevalence of patients harboring a mutation in *KCNJ5* is 43% as observed in a meta-analysis of 1,636 patients with an APA from 13 studies [35]. However, the prevalence varies from 34% to 77% depending on the study population and shows highest incidence in Asian centres [25, 35–38]. The second highest prevalence of somatic variants occurs in *CACNA1D* (14–27%) in the majority of reports to date [25, 39]. However, in patients of recent African ancestry, *CACNA1D* is the most frequently mutated gene (42% prevalence) followed by *KCNJ5* (34% prevalence) [40]. Somatic mutations in *ATP1A1* and *ATP2B3* are present at a lower incidence (5-13% and 1–10%, respectively) [25, 39] and are quite rare occurrences in *CLCN2* or *CACNA1H* with a frequency of 1% [25, 41].

APAs with *KCNJ5* mutations are reported to be more prevalent in female patients, have a larger size, and are associated with specific peripheral venous plasma profiles [25, 35–37]. The first described PA-associated variants in *KCNJ5* were the predominant APA G151R and L168R mutations and a germline variant encoding a T158A substitution [26, 42]. All mutations are located in or close to the selectivity filter of the *KCNJ5* channel and lead to a loss of channel selectivity that facilitates sodium influx [26, 42]. G151R has also been described as a germline mutation, which like T158A, induces the formation of massive adrenocortical hyperplasia [26, 43]. In contrast, another germline mutation, G151E, appears associated with a milder clinical phenotype and with normal appearing adrenals at computed tomography imaging [42, 43]. These features can be accounted for by the remarkably higher Na^+ conductance of the G151E mutated KCNJ5 channel that promotes cell death by osmotic shock and thereby prevents the dysregulated cell proliferation observed in hyperplastic adrenals with G151R and T158A variants [26, 43].

Histopathologic evaluation of *KCNJ5*-mutated APAs reveals lower expression of CYP11B2 and higher expression of CYP11B1 compared with adenomas without a *KCNJ5* mutation [44].

The observed co-localization of CYP11B2 with CYP17A1 in APAs with such mutations [45] might explain the synthesis of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol [46, 47] as these were found in several studies to be increased in patients with *KCNJ5*-mutated APAs compared with APAs without *KCNJ5*-mutations [39, 48, 49].

Mutations in *CLCN2* and *CACNA1H* are found both as germline and sporadic variants [30–32, 41, 50]. The identification of variants in these genes in APAs were only recently identified, in 2019 and 2020, respectively, so that currently not many cases have been described to date [25, 41]. Nonetheless, CLCN2-G24D as well as CACNA1H-I1430T have both been shown to cause increased aldosterone secretion from adrenocortical cells *in vitro* [30, 41]. Similarly, ATP1A1-G99R, -L104R, and -V332G proved to alter the channel gating mechanism, lead to cell membrane depolarization and induce increased expression of *CYP11B2* [28, 51]. However, not all identified variants in aldosterone-driver genes have been confirmed for their pathogenicity as demonstrated by a electrophysiological study that revealed the non-pathogenic role of the CACNA1D-M1354I variant [52].

Two different models for APA formation have been postulated and widely discussed. One is the two-hit-model in which two independent mutations have to occur - one with a proliferative effect and a second inducing increased aldosterone biosynthesis [53]. The second model hypothesizes the transition of APAs from APMs named possible APM-to-APA transitional lesions (pAATLs) based on the findings of Nishimoto et al. who reported micro-APAs consisting of lipid-rich cells within APM-like structures located in the ZG that harbour aldosterone-driver mutations [54]. Interestingly, APMs show different proportions of mutations in known aldosterone-driver genes than APAs harbouring predominantly mutations in *CACNA1D* or *ATP1A1*, but never or only very rarely in *KCNJ5* [55–57].

1.1.5 Current Difficulties in Subtype Classification

The Primary Aldosteronism Surgical Outcome (PASO) consensus, established in 2017, defined criteria to assess outcomes of patients at 6–12 months after adrenalectomy for unilateral PA [58]. These PASO criteria are divided in clinical and biochemical outcomes and classify the blood pressure response and the correct diagnosis of unilateral PA, respectively [58]. Both the clinical and biochemical categories are assessed as complete, partial or absent success [58]. The clinical outcome is evaluated based on the blood pressure values and the use of

antihypertensive medication, while the biochemical outcome refers to the ARR and plasma potassium levels [58]. Complete biochemical success is assigned to patients who displayed a normalization of the ARR and a correction of hypokalaemia (if present pre-surgery) indicating a correct diagnosis of unilateral PA resolved by the removal of the diseased adrenal [58]. Patients with a partial or absent biochemical outcome imply disease persistence presumably caused by bilateral asymmetrical aldosterone production [58].

AVS is the gold standard approach recommended by the Endocrine Society Guideline for subtype differentiation of unilateral from bilateral PA [8]. In an international multicentre study, the use of AVS for subtype diagnosis reduced the proportion of patients with incomplete biochemical cure after unilateral adrenalectomy from 20% to 7% compared with patients subtyped based on computed tomography scanning [59]. Segmental selective AVS of each central and tributary adrenal vein is a further highly technical development of the procedure that is used only in Japan [60, 61]. This approach allows finer mapping of the source of aldosterone overproduction for eventual partial adrenalectomy [60, 61]. However, AVS is a patient unfriendly, minimal invasive procedure associated with high costs and requires experienced interventional radiologists. For these reasons, alternatives are sought to replace AVS in at least some patients. In addition, the standard AVS approach identifies the dominant adrenal with lateralized highest aldosterone secretion, but neglects cases in which the contralateral, non-dominant adrenal also has abnormally high aldosterone secretion. Such patients with asymmetrical bilateral aldosterone production can be misdiagnosed with AVS as unilateral instead of bilateral PA and thus show persistent aldosteronism after unilateral adrenalectomy [58].

Subtype differentiation of PA using functional imaging with specific molecular tracers in positron emission tomography-computed tomography, such as ^{11}C-metomidate against the enzymes CYP11B1 and CYP11B2 [62, 63], or 68Ga-pentixafor against the chemokine receptor CXCR4 (that is positively correlated with CYP11B2 expression in APAs [64]), has shown initial promise for the detection of APAs. However, the limited spatial resolution allows only reliable identification of APAs >10–15mm in diameter [63, 64] which limits the wider applicability of this technique for the diagnostic management of PA [19]. Peripheral venous steroid profiling shows particular usefulness for subtype differentiation [65], especially for the identification of APAs with a *KCNJ5* mutation [48, 66](see 1.1.6).

1.1.6 Molecular Profiling of Primary Aldosteronism

1.1.6.1 Steroid Profiling

Analysis and quantification of steroids is complex because steroids often have the same molecular mass resulting in equivocal chromatograms when using mass spectrometry only. Thus, time-resolved analysis is required to separate the peaks of these very similar steroids by using either liquid or gas chromatography prior to mass spectrometry. By combining these two techniques, steroids from plasma of peripheral venous blood or adrenal venous blood as well as urine from patients with PA were analysed by either liquid chromatography-tandem mass spectrometry (LC-MS/MS) or gas chromatography-mass spectrometry in several studies over recent years.

The potential utility of the hybrid steroids, 18-oxocortisol and 18-hydroxycortisol, have been investigated for subtype differentiation in PA. Both are significantly elevated in peripheral plasma of patients with an unilateral APA compared with patients with bilateral adrenal hyperplasia [67, 68] and are higher in APAs with *KCNJ5* mutations compared to those without [48]. However, to predict the subtype of PA, the measurement of a combination of different steroids was shown to be a better tool than using solitary steroids. Eisenhofer et al. correctly classified 80% of patients with an APA or bilateral adrenal hyperplasia using a panel of 12 steroids in peripheral venous blood samples [65]. Moreover, Williams et al. demonstrated that a fingerprint of seven steroids in peripheral venous blood of patients with an APA was sufficient to correctly classify these adenomas with an accuracy of 92% according to their genotype [48]. In a recent multicentre clinical trial, steroid profiling integrated with machine learning technologies has been shown to be potentially useful to screen for patients with a unilateral APA with a *KCNJ5* mutation [66]. Overall, steroid profiling is highly promising as a diagnostic tool in the management of patients with PA.

1.1.6.2 Metabolomic Profiling

Matrix-Assisted Laser Desorption/Ionization - Mass Spectrometry Imaging (MALDI-MSI) can be applied for *in situ* metabolomic profiling of tissue sections. The technology is based on a matrix that is applied on tissue sections [69]. A laser beam, absorbed by the matrix, initiates the matrix to take up the analytes from the tissue and facilitate the desorption and

ionization step for the following mass spectrometry [69]. Matrix composition is chosen based on which target analytes are investigated and the molecular weight of these target analytes determines the best mass spectrometry imaging approach [69]. Through this technology, the tissue itself remains intact and can be used for a subsequent immunohistochemical staining that can be imaged and integrated with MALDI-MSI data [69]. This approach enables the combination of the morphological phenotype with the identification and spatial visualization of thousands of analytes such as lipids, proteins, peptides, steroids, drugs, and cell metabolites within a single tissue section or specific region of interest within a section [69].

Recent advances in methodology by Prof. Axel Walch's group at the Helmholtz Zentrum München, Germany have demonstrated the application of this technology to fresh frozen tissue as well as FFPE tissue sections [70, 71]. The use of Fourier-Transform Ion Cyclotron Resonance (FT-ICR) in MALDI-MSI enables the high mass resolution and accuracy mandatory for investigating FFPE tissue sections [71]. The first MALDI-MSI of normal human adrenal glands in 2018 [72] was soon thereafter followed by imaging analyses of APAs [73] and APMs [74]. Using fresh frozen tissue sections, Sugiura et al. demonstrated the presence of aldosterone as well as the hybrid steroid 18-oxocortisol in both, APAs and APMs [74]. Moreover, Murakami et al. investigated the metabolomic profiles of APAs from FFPE tissue sections in regard to their genotypes [73]. Unguided hierarchical cluster analysis initially could not identify distinct metabolic signatures from the whole sample set, but the pairwise comparison of APAs with *KCNJ5 versus CACNA1D* mutations successfully classified the two subsets of APAs according to genotype [73].

Because APMs also carry aldosterone-driver mutations, although in divergent proportions than APAs [55], and have been additionally postulated to transform into APAs [54] (see 1.1.4), this technology could help to further examine aldosterone-producing lesions, specifically APMs, in patients with PA to understand the molecular pathophysiology of this complex endocrine disorder.

1.1.6.3 Genotyping

Tremendous advances in identifying the genetic background of PA are attributed to NGS approaches of which whole exome sequencing and Ion-Torrent based targeted NGS are currently the most prominently used methods in this clinical field. Most disease-causing mutations are

observed in exonic (protein-coding) regions of the human genome, although the relevance of non-coding DNA in disease has gained increasing importance over recent years [75] including a potential role for microRNAs in PA pathophysiology [76]. As soon as more than a few genes need to be investigated, Sanger sequencing is not practicable in terms of costs and time [77]. Economical alternatives in such cases are whole exome sequencing or Ion-Torrent based targeted NGS. Although there is a trend towards whole genome sequencing [75], the costs for whole exome sequencing are comparably lower [78] and datasets are more compact and easier to manage enabling easier and faster analysis.

Whole exome sequencing has unravelled gene variants that cause somatic forms of PA. These mutations are in the *KCNJ5* gene, predominantly G151R and L168R substitutions [26], mutations in *ATP1A1*, *ATP2B3* and *CACNA1D* [27, 28], and a few cases of *CLCN2* [31, 32] and *CACNA1H* [30, 50] mutations. The identification of potential disease causative variants in the majority of APAs shifts the emphasis from Sanger sequencing to the increased use of targeted NGS due to the improved success of variant detection [25, 39].

1.2 Aims

The research projects of this dissertation aimed to investigate the pathophysiology of PA by examining adrenal immunohistochemistry and molecular histopathology of patients diagnosed with PA.

- **Aim I**: To establish the steroidobolomics profiles associated with the histopathology of PA (Publication I)
- **Aim II**: To determine the metabolomics profiles associated with the histopathology of PA (Publication II)
- **Aim III**: To investigate clinical, histopathological and genetic correlates of a 3-year prospective series of surgically-treated patients with PA (Publication III)

The respective projects of these aims resulted in three publications. Of note, the HISTALDO consensus [19] was published during the time of these PhD projects. Thus, publications I and II used a slightly different nomenclature of aldosterone-producing lesions in the resected

adrenals compared with the third publication, which was entirely based on the HISTALDO consensus [19].

1.3 Major Findings

Publication I with the title *"Immunohistopathology and Steroid Profiles Associated With Biochemical Outcomes After Adrenalectomy for Unilateral Primary Aldosteronism"*, published 2018 in Hypertension, included 95 patients operated for unilateral PA collected from 9 international referral centres. Clinical and biochemical outcomes were assessed according to the PASO criteria [58]. Forty-three patients with absent or partial biochemical success were matched for sex and age with 52 patients with complete biochemical success [79]. HE stainings and CYP11B2-immunohistochemistry were performed on all adrenals [79]. Steroid profiling was conducted in collaboration with the group of Prof. Eisenhofer (Institut für Klinische Chemie und Laboratoriumsmedizin, Universitätsklinikum Carl Gustav Carus an der Technischen Universität Dresden, Germany), using LC-MS/MS. Peripheral venous plasma steroid profiling was performed on a subset of patients and compared with data from 27 patients diagnosed with bilateral PA [79]. Immunohistopathology assessment showed that patients with complete biochemical success have a higher prevalence of a solitary APA ($P<0.001$), while patients with incomplete biochemical cure have a higher prevalence of adrenocortical hyperplasia ($P=0.004$) [79]. Predictive models showed accurate classification of the histopathological phenotypes according to the adrenal steroid measurements [79]. Decision tree analysis as well as linear discriminant analysis using a receiver-operator curve demonstrated an association of the adrenal steroid profiles with biochemical outcomes (complete *versus* partial+absent) and the diagnosis of bilateral PA [79].

Publication II with the title *"Mass Spectrometry Imaging Establishes 2 Distinct Metabolic Phenotypes of Aldosterone-Producing Cell Clusters in Primary Aldosteronism"*, published 2020 in Hypertension, was performed in collaboration with the group of Prof. Dr. med. Axel Walch (Research Unit Analytical Pathology, German Research Center for Environmental Health, Helmholtz Zentrum München, Germany) using a Matrix-Assisted Laser Desorption/Ionization Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry Imaging

(MALDI-FT-ICR MSI) platform recently established for FFPE tissue sections [70]. The study included 6 APAs and 27 APMs from 10 adrenals from patients diagnosed with unilateral PA at the Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munich, Germany, that were assessed by CYP11B2 immunohistochemistry [80]. One of these adrenals showed both an APA and 3 APMs in the adjacent adrenal cortex [80]. Metabolomic profiles of all APAs and APMs were analysed and related to their CYP11B1/CYP11B2 double immunofluorescence stainings [80]. Additionally, genotypes were determined of all APAs and 7 APMs after metabolomic profiling using a specifically established CYP11B2-guided Sanger sequencing approach [80]. Heat-map based cluster analysis of the 27 APMs showed a separation into two distinct subgroups of which APMs in subgroup 1 were densely clustered and clearly distinguishable from those in subgroup 2 and the APAs [80]. In contrast, APM subgroup 2 displayed a similar metabolic fingerprint compared to APAs [80]. Interestingly, APMs found in the same adrenal were always classified into the same APM subgroup [80]. Clinical data indicated a younger age in patients with APM subgroup 1 compared with APM subgroup 2 and additionally a lower ARR compared with the APA group [80]. Double immunofluorescence demonstrated no apparent differences between the two APM subgroups [80]. Genotyping revealed heterogeneous mutations in a heterogeneous group of aldosterone-driver genes in APAs and in APM subgroups 1 and 2 with the exclusion of *KCNJ5* mutations in both groups of APMs [80].

Publication III with the title *"Single Center Prospective Cohort Study on the Histopathology, Genotype and Postsurgical Outcomes of Patients With Primary Aldosteronism"*, published 2021 in *Hypertension*, highlights the clinical, histopathological and genetic correlates of a 3-year prospective series of surgically-treated patients for PA. The study included 60 patients consecutively operated for PA between 2016 and 2018 at the Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munich, Germany. CYP11B2 immunohistochemistry and HE staining were performed on sections from all adrenal FFPE blocks and evaluated according to the recently established HISTALDO consensus [19]. Follow-up data were obtained from 54 of 60 patients to evaluate clinical and biochemical outcomes according to the PASO criteria [58]. AVS parameters were available for 54 patients. Immunohistochemistry revealed classical histopathological phenotypes showing a solitary APA or APN without (n=19) or with MAPM (n=23) or APDH (n=3) in the adjacent adrenal cortex in 45 of 60

(75%) patients [81]. The remaining 15 adrenals (25%) displayed nonclassical histopathology with a solitary APA or dominant APN absent, but with MAPM or MAPN (n=12) or APDH (n=3) [81]. From AVS results, we showed that patients with a nonclassical histopathology had higher aldosterone secretion from the contralateral adrenal gland compared with patients with the classical histopathology [81]. In addition, those patients were less often biochemically cured after surgery compared with patients with a solitary APA or dominant APN (P=0.002) while clinical outcomes did not differ between the two groups (P=0.224) [81]. Genotyping was performed from all solitary APA or dominant APN using DNA extracted from CYP11B2-positive regions of FFPE tissue sections and sequenced by Sanger sequencing or sent for exome sequencing to Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany) with subsequent analysis by the Ingenuity Variant Analysis (Qiagen) [81]. The genotype of samples with no mutation detected were validated by Ion-Torrent-based targeted NGS by the group of Prof. Dr. William E. Rainey (Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan, USA). We identified mutations in aldosterone-driver genes in 85% (35 of 41) of APAs or dominant APNs with a prevalence of 56% for *KCNJ5* mutations, which were not associated with clinical or biochemical outcomes [81].

1.4 Significance and Future Perspectives

The findings of these publications are contemporary in the research area of PA and disclosed novel insights into the clinical phenotype and molecular histopathology of the disease. The major strength of all publications is the collection of follow-up data as well as AVS parameters from a high percentage of Munich patients. Post-surgical clinical and biochemical outcomes were uniformly assessed according to the international PASO criteria [58]. For every project, we evaluated the histopathology of sections of all adrenal FFPE blocks from each Munich patient by CYP11B2-immunohistochemistry and HE staining which is of high importance to assess all fragments of the diseased adrenal and visualize potential aldosterone-producing lesions throughout the resected adrenal.

The power of the first publication is the multicentre design and the high number of relatively rare adrenal samples collected from patients with incomplete biochemical outcomes

that were carefully matched for sex and age with patients with complete biochemical success to reduce the effects of bias [79]. Publication II stands out with its combinatory characterization of APMs by metabolomic profiling, immunofluorescence staining, as well as genetic analysis. The third publication was one of the first groups to assess adrenal immunohistochemistry based on the international HISTALDO consensus [19] combined with application of the PASO criteria [58] for the evaluation of postsurgical outcomes.

Correctly classifying the histopathological phenotype proved of high importance to demonstrate that patients with the nonclassical histopathology without a solitary APA or dominant APN displayed increased contralateral aldosterone secretion at AVS compared with patients with classical histopathology with a solitary APA or dominant APN suggesting bilateral asymmetrical aldosterone production [81]. Moreover, we could show that steroid profiles are associated with adrenal histopathology and biochemical outcomes [79]. Together, these findings highlight the heterogeneous histopathology of PA, the potential influence of histopathology on patient outcomes, and the role it may play in guiding follow-up care.

Our approach of CYP11B2-guided sequencing ensures the capture of mutations in aldosterone-driver genes specific to the CYP11B2-positive regions of interest. The additional use of NGS methods enabled to increase the number of identified aldosterone-driver mutations as reported in previous publications.

We demonstrated for the first time the presence of two distinct metabolomic subgroups of APMs with potential different pathophysiological impact [80]. The functional significance of the two metabolomic phenotypes is unclear but future metabolomic studies using a larger number of APMs integrated with biochemical outcome data are planned to dissect their putative respective roles in unilateral and bilateral forms of PA. Further understanding of the biology of APMs could be achieved by combining metabolomics with analysis of transcriptome profiles. State of the art technologies such as spatial transcriptomics enable gene expression profiling within the context of histopathology or protein expression determined by immunofluorescence. Application of *in situ* metabolomics and spatial transcriptomics technologies to resected adrenals from patients with PA would allow in depth profiling of APM subgroups and open up new perspectives in the pathophysiology of PA.

2 My Contribution

For **Publication I** with the title *"Immunohistopathology and Steroid Profiles Associated With Biochemical Outcomes After Adrenalectomy for Unilateral Primary Aldosteronism"* published in Hypertension 2018, I compiled and managed the dataset of the patient cohort including matching for sex and age. Immunohistochemistry for CYP11B2 and HE stainings of the adrenals and genotype analysis was predominantly performed by me. All statistical analyses, except receiver-operator curves and decision tree analyses, were conducted by myself. In addition, I provided the draft of the manuscript and prepared figures and tables.

For **Publication II** with the title *"Mass Spectrometry Imaging Establishes 2 Distinct Metabolic Phenotypes of Aldosterone-Producing Cell Clusters in Primary Aldosteronism"* published in Hypertension 2020, I performed all initial CYP11B2 immunohistochemistry for adrenal sample selection and performed CYP11B1 and CYP11B2 double immunofluorescence on the selected samples. I was responsible for establishing CYP11B2-guided Sanger sequencing of APMs in our laboratory which I used for this study. Finally, I performed genotyping of all adrenal samples in this study.

For **Publication III** with the title *"Single Center Prospective Cohort Study on the Histopathology, Genotype and Postsurgical Outcomes of Patients With Primary Aldosteronism"*, I did the majority of CYP11B2-immunohistochemistry and HE stainings and analysed the histopathology together with Dr. Tracy Ann Williams. I extracted DNA from CYP11B2-positive regions of FFPE tissue sections and performed Sanger sequencing of APAs and APNs. Additionally, I extracted DNA from fresh frozen tumour tissue, leukocytes or CYP11B2-positive regions of FFPE tissue sections for exome sequencing or Ion-Torrent-based targeted NGS. Cell transfection and functional analysis of the CLCN2 variant were entirely done by me. Data analysis from Sanger sequencing and exome sequencing as well as the database management with clinical and biochemical data including its statistical analyses completes my contribution to this project. Finally, I prepared the figures, tables and wrote the manuscript.

3 Publication I

Immunohistopathology and Steroid Profiles Associated With Biochemical Outcomes After Adrenalectomy for Unilateral Primary Aldosteronism

Authors: Lucie S Meyer, Xiao Wang, Eva Sušnik, Jacopo Burrello, Alessio Burrello, Isabella Castellano, Graeme Eisenhofer, Francesco Fallo, Gregory A. Kline, Thomas Knösel, Tomaz Kocjan, Jacques W.M. Lenders, Paolo Mulatero, Mitsuhide Naruse, Tetsuo Nishikawa, Mirko Peitzsch, Lars C. Rump, Felix Beuschlein, Stefanie Hahner, Celso E. Gomez-Sanchez, Martin Reincke, Tracy Ann Williams

Hypertension. 2018;72:650-657. DOI: 10.1161/HYPERTENSIONAHA.118.11465

4 Publication II

Mass Spectrometry Imaging Establishes 2 Distinct Metabolic Phenotypes of Aldosterone-Producing Cell Clusters in Primary Aldosteronism

Authors: Na Sun, Lucie S Meyer, Annette Feuchtinger, Thomas Kunzke, Thomas Knösel, Martin Reincke, Axel Walch, Tracy Ann Williams

Hypertension. 2020;75:634-644. DOI: 10.1161/HYPERTENSIONAHA.119.14041

5 Publication III

Single-Center Prospective Cohort Study on the Histopathology, Genotype, and Postsurgical Outcomes of Patients With Primary Aldosteronism

Authors: Lucie S Meyer, Laura Handgriff, Jung Soo Lim, Aaron M Udager, Isabella-Sabrina Kinker, Roland Ladurner, Moritz Wildgruber, Thomas Knösel, Martin Bidlingmaier, William E Rainey, Martin Reincke, Tracy Ann Williams

Hypertension. 2021;78:738–746. DOI: 10.1161/HYPERTENSIONAHA.121.17348

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