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Summary

Chapter 1 provides a general introduction and defines the aims of the present thesis. The function of the adrenal glands is described with a focus on the biosynthesis of steroid hormones in the adrenal cortex and evaluation of steroidogenesis with urinary steroid profiling. In addition, the clinical evaluation of adrenal tumors is described. The last section focuses on the preoperative treatment of patients with pheochromocytomas or sympathetic paragangliomas (PPGL). The overall aim of this thesis is to improve diagnostic strategies that intend to differentiate clinically relevant adrenal tumors from those without clinical consequence and to optimize preoperative treatment in order to prevent hemodynamic instability during surgical resection of a PPGL.

Part I of this thesis focused on adrenal steroidogenesis and its relationship with lipoproteins. In Chapter 2 we described the validation of urinary steroid profiling using a newly developed gas chromatography with tandem mass spectrometry detection method (GC/MS-MS). This new method demonstrated excellent results for all validation parameters and the specificity was improved compared to the previously applied GC-MS method. A main advantage of this new method is a reduction of the analysis time to less than 24 hours, making it suitable for high-throughput analysis. Reference values for all 33 analyzed steroid metabolites were determined in a population of 240 healthy subjects recruited from the Lifelines cohort stratified by age and sex. In addition, 40 women using oral contraceptive pills (OCP) were analyzed separately. The results demonstrated that the urinary excretion of almost all steroid metabolites is affected by age and sex, underscoring the need for age and gender specific reference values. Moreover, the use of OCP resulted in a lower excretion of progesterone and androgen metabolites and this factor should be taken into account in daily clinical practice.

In Chapter 3 we determined the relationship between total glucocorticoid production (TGP) and plasma HDL-C levels, in view of previous findings suggesting that adrenal function may be attenuated in men with genetically determined low levels of plasma high density lipoprotein cholesterol (HDL-C). TGP was estimated as the sum of glucocorticoid metabolites that were determined in a 24-hour urine collection using GC/MS-MS. We analyzed these variables in 240 healthy subjects recruited form the Life Lines cohort who were stratified by age and sex. TGP tended to be increased in subjects with a low plasma HDL-C concentration according to NCEP-ATPIII criteria. Univariate analysis demonstrated that TGP was inversely
correlated with HDL-C. Multivariable linear regression analysis demonstrated that TGP was still inversely related to HDL-C after adjustment for a variety of metabolic syndrome components. These data do not support the hypothesis that adrenal function is attenuated in subjects with low plasma concentrations of HDL-C as previously suggested.

In Chapter 4 we measured lipoprotein particle concentrations in 23 patients with primary aldosteronism. Blood samples collected during an adrenal vein sampling procedure from the inferior vena cava (IVC) and both adrenal veins were analyzed. We found that plasma HDL-C and particle concentrations of HDL and of LDL were not lower in the adrenal veins compared to the IVC, whereas ApoB tended to be lower in the adrenal vein samples. In a subset of 13 patients with an aldosterone producing adenoma in whom we expected to find the highest uptake, it was found that plasma ApoB concentrations were lower with a trend towards lower LDL particle concentrations in the adrenal vein. These observations suggest that cholesterol derived from circulating LDL particles may contribute to adrenal steroidogenesis, and that adrenal venous sampling is a feasible model to determine uptake of lipoproteins in the adrenal glands.

In Part II of this thesis we focused on diagnostic strategies for PPGL. In Chapter 5 we performed a nationwide pathology study to identify all histopathologically confirmed cases of PPGL diagnosed between 1995 and 2015. A total of 1493 patients with PPGL were identified. We found that the age-standardized incidence rates for PPGL increased during the study period. Concomitantly, pheochromocytoma size decreased and age at diagnosis increased. Additionally, we conducted a systematic review of the literature in which we identified only three papers reporting on nationwide incidence rates of PPGL between 1949 and 1981 in North-West European countries. A comparison of these incidence rates showed that the observed increase in incidence started already several decades ago. These results might in part reflect the changing clinical practice with augmented use and improved accuracy of imaging and biochemical tests for detecting PPGL.

Chapter 6 concerned a retrospective multi-center study in which unenhanced CT-scans of 214 patients harboring 222 histopathologically confirmed pheochromocytomas were reevaluated by two independent radiologists. We found that only one pheochromocytoma demonstrated an unenhanced attenuation value ≤10 Hounsfield Units (HU). Moreover, the interobserver consistency and concordance were excellent. These data support the hypothesis
that biochemical testing to rule out a pheochromocytoma in patients with an adrenal incidentaloma is only indicated when the unenhanced attenuation value is $>10$ HU, and suggest that attenuation measurement for this purpose is well suited for implementation in general clinical practice.

In Chapter 7 we confirmed the results of chapter 6 in a systematic review and meta-analysis, including 1167 pheochromocytoma cases from 31 studies. We found that the proportion of pheochromocytomas with an unenhanced attenuation value $>10$ HU was $0.990$ (95% CI: 0.984-0.995). The negative predictive value was estimated to be close to 100%. A new diagnostic algorithm in which biochemical testing to exclude a pheochromocytoma is obviated in patients with an adrenal tumor and unenhanced attenuation value $\leq 10$ HU was proposed. A probabilistic sensitivity analysis that modeled the costs of this diagnostic algorithm demonstrated a modest cost reduction compared to current diagnostic practice.

Part III of this thesis was focused on optimizing perioperative hemodynamic stability in patients with PPGL. In Chapter 8 we described the development and internal validation of the hemodynamic instability score (HI-score). The premise of the HI-score is that both hemodynamic variables as well as interventions aiming to normalize hemodynamic variables (i.e. cardiovascular medication and fluid therapy) are important elements of overall hemodynamic instability. In a development cohort, consisting of patients that were expected to represent the entire hemodynamic instability spectrum, normalized threshold values of all hemodynamic and intervention variables were determined to assign scores. Subsequently, these scores were applied to the validation cohort in which we demonstrated that the HI-score was significantly different between surgical procedures associated with a low or high degree of hemodynamic instability. The HI-score provides a clinical tool which shows promise for future applications in both patient management and clinical research following further external validation.

Chapter 9 involved the first randomized controlled trial that compares the efficacy of two $\alpha$-receptor antagonists in the preoperative treatment of patients undergoing resection of a PPGL. Patients were randomized between pretreatment with either doxazosin or phenoxybenzamine. The primary endpoint, defined as the cumulative percentage of intraoperative time with a systolic blood pressure $>160$ mmHg or a mean arterial pressure $<60$ mmHg, was not different between treatment groups. The overall degree of hemodynamic instability, assessed by
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the HI-score, was higher in the doxazosin group. No differences in mortality or cardiovascular complications rates were observed between both treatment arms. Of note, patients with a cardiovascular complication had a significantly higher degree of hemodynamic instability. Based on secondary endpoints, the results of this trial suggest a greater efficacy of phenoxybenzamine in preventing intraoperative hemodynamic instability.
General discussion

Cholesterol trafficking into and within the steroidogenic cell is a complex process to study, since there are multiple pathways by which cholesterol can be delivered to the mitochondria for further processing (3). Cholesterol in adrenal cortical cells can be derived from i) de novo synthesis in the endoplasmic reticulum, ii) storage in intracellular lipid droplets, or iii) circulating lipoproteins. Studies from the 1960s and 1970s using radiolabeled cholesterol demonstrated that circulating cholesterol is the most important contributor to adrenal steroidogenesis (4). Both the LDL-receptor, that internalizes the entire LDL-particle, and the SRB1-receptor, that enables cholesterol import from the HDL-particle into the cell, are present on human steroidogenic cells (3-6). It is therefore conceivable that both HDL and LDL may play a role in cholesterol delivery needed for adrenal steroid biosynthesis. In vivo studies that investigated the relative contributions of both these classes of lipoproteins predominantly have been performed in rodents (4). However in rodents, HDL serves as the predominant lipoprotein class with lower levels of LDL compared to humans, in whom LDL is the predominant class with lower levels of HDL. Findings from these animal experiments, therefore, do not necessarily hold true for humans. In an attempt to directly determine the uptake of lipoprotein particles we used adrenal venous sampling in patients with primary aldosteronism. Of note, blood flow in the adrenal glands as well as concentrations of circulating lipoproteins are relatively high compared to steroid output, even in the context of ACTH stimulation (7). The rate at which cholesterol is extracted from circulating lipoproteins by the adrenal glands is, therefore, presumably low. Despite these challenges we were able to detect a consistent uptake of apoB and a trend towards uptake of LDL-particles. We regard this as a proof of concept and consider this approach feasible to assess lipoprotein uptake in humans. No evidence was found for HDL-C uptake or for a change in HDL particle size. The latter would have been expected given the mechanism of SRB1-mediated cholesterol uptake that directs the cholesterol cargo of HDL particles into the adrenal cortical cells leaving the HDL-particle itself intact. Of note in a subsequent study, non-diabetic patients derived from the same cohort of hyperaldosteronism patients, were found to have significantly lower levels of LDL particle and HDL particle concentrations compared to normotensive and hypertensive subjects (8). The mechanism that is responsible for this apparently unique lipoprotein profile in patients with primary aldosteronism is currently unclear.

The relationship between concentrations of circulating lipoproteins and steroid hormone production in humans is predominantly derived from patient populations
with certain genetic alterations in cholesterol metabolism and trafficking. For example, patients with abetalipoproteinemia or defects in the LDL receptor pathway were demonstrated to have normal basal cortisol production, but a mildly attenuated response to ACTH (9,10). This suggests that cholesterol supply from \textit{de novo} synthesis or from circulating HDL is sufficient to ensure adequate cortisol production under basal conditions. In contrast, basal steroidogenesis was suggested to be attenuated in male patients with genetically low HDL-levels but responses to ACTH stimulation were normal (1). In women with genetically determined low HDL-levels the adrenal function was, however, normal (11). Additionally, both basal and stimulated adrenal function was decreased in a family with a mutation in the SRB1 receptor that facilitates extraction of cholesterol from HDL-particles into the steroidogenic cell. Together, these data suggest an important role for the HDL-SRB1 pathway in basal and stimulated steroidogenesis, but the evidence is somewhat equivocal. It should be noted that various methods were used to quantify steroid production. By application of a more precise technique we found a trend towards an increased glucocorticoid production in subjects with non-genetically determined low HDL levels. Thus, it seems likely that low HDL-C levels do not contribute to decreased glucocorticoid production under basal circumstances. Possibly, even low levels of HDL-C are sufficient to utilize the HDL-SRB1 pathway or the LDL pathway is utilized simultaneously. Further employment of the adrenal venous sampling model to compare subjects with the aforementioned genetic alterations under both basal and stimulated conditions may give further direction to this research question. Additionally, super- selective adrenal venous sampling might reduce venous dilution significantly (12). Alternatively, synthetic lipoproteins filled with, for example, radiolabeled cholesterol could be used to study adrenal lipoprotein utilization \textit{in vivo} (13,14).

Overall our findings suggest that LDL might be important for steroidogenesis after ACTH stimulation, but we cannot exclude the possibility that HDL contributes as well. From an evolutionary point of view, it could be envisaged that multiple pathways are in place for such a vital physiological function as steroid biosynthesis and that, therefore, both LDL and HDL particles can be used to supply cholesterol to the adrenal glands.

Adrenal incidentalomas are by definition a direct consequence of the ever increasing utilization and improving performance of imaging techniques (15,16). This clear trend in the clinical application of various imaging modalities results in
the detection of more and smaller sized adrenal incidentalomas. Distinguishing between adrenal tumors that need specific treatment and benign non-functioning adenomas is, has therefore become an increasingly relevant clinical problem. When discussing the diagnostic approach of patients with an adrenal incidentaloma it is foremost important to consider how often an adrenal tumor has clinical consequences. In 2016, more than 1.7 million CT-scans were performed in the Netherlands (16). The adrenal glands are visualized in about two thirds of these. Assuming a 3-10% prevalence of adrenal incidentalomas as reported in radiological studies, the number of patients with an adrenal incidentaloma is quite high (17,18). Reliable incidence rates are lacking, but it can be estimated that: 8500 patients with an adrenal incidentaloma should be annually identified in The Netherlands assuming that 1 in 4 CT-scans is performed in a unique patient and the adrenal incidentaloma frequency is 3%. A systematic review of the literature demonstrated that adrenocortical carcinomas, pheochromocytomas and metastases represent 8% (range: 1.2-11%), 7% (range: 1.5-14%) and 5% (range: 0-18%), respectively, of all adrenal incidentalomas.(19) Obviously, the frequency of aforementioned clinical relevant etiologies are not in agreement with their known incidence rates considering the prevalence of adrenal incidentalomas. For example, the annual incidence rate of adrenocortical carcinoma is just over 1 per million person-years (20). Assuming an adrenal incidentaloma frequency of 8500, 8% of which represent an adrenocortical carcinoma, then the incidence rate of adrenocortical carcinoma would be 40 per million person-years. The same line of reasoning applies to pheochromocytomas. We demonstrated that the incidence rate of pheochromocytomas is increasing over the years, but this disease should still be considered a rare disease. During the entire observation period, the incidence rate was the highest and increased the fastest in patients older than 50 years. This suggests an important contribution of incidental discovered pheochromocytomas, since this age group is most frequently scanned. However, the incidence rate we found is not even nearly in agreement with the 7% frequency of pheochromocytomas in adrenal incidentalomas. Of note, it is conceivable that not all pheochromocytomas are currently detected. Especially, oligosymptomatic pheochromocytomas are often discovered as adrenal incidentaloma and are therefore more likely to remain undetected despite a larger tumor size and higher metanephrines compared to hereditary and symptomatic cases (21).

Overestimation of the frequency of clinical significant adrenal incidentalomas is likely the result from several kinds of methodological shortcomings, in particular selection bias in the reported studies (22). About half of adrenal incidentalomas
that are visualized on CT are not mentioned in the radiology report (23). In addition, larger lesions are more often reported in specific terminology by the radiologist and these patients are more frequently referred for further analysis compared to non-specifically reported lesions (24). In real-life clinical practice, however, only 14% of patients with an adrenal incidentaloma that was reported in specific terminology underwent a complete biochemical and radiological workup (24). The smaller non-specifically reported lesions are less likely to be clinically relevant but are underrepresented in the majority of retrospective studies. However, size alone is a suboptimal predictor of hormonal hypersecretion (25). Therefore, the proportion of patients with a clinical relevant adrenal incidentaloma is overestimated but at the same time clinical relevant adrenal incidentalomas are currently missed. This may significantly influence the performance of various diagnostic tests that are applied in patients with an adrenal incidentaloma in daily clinical practice.

Many diagnostic algorithms for adrenal incidentalomas have been published over the years (19,26-33). A high diagnostic burden to evaluate the nature and hormonal function of adrenal incidentalomas is a common denominator of these guidelines. The level of evidence that supports these recommendations is in general either low or very low. The major factor of the low level of evidence is the retrospective design of the majority of studies that were taken into account to generate the guidelines. Nowadays, the overall trend in the diagnostic approach is towards less diagnostic tests and a shorter follow-up time (19). This makes sense from the perspective that at least 70%, and probably a much higher percentage, of all adrenal incidentalomas can be classified as benign non-functioning adenomas. This thesis contributes to the changing view in diagnostic approach by demonstrating that determination of metanephrines can be obviated in patients harboring an adrenal incidentaloma with an unenhanced attenuation value ≤10 Hounsfield Units (HU). Admittedly, this recommendation is also based mostly on retrospective data. However, selection bias appeared not to be a major confounder in our meta-analysis since we only looked to determine the sensitivity of the 10 HU cut-off value and the results, therefore, seem to be robust. Prospective validation would further strengthen this recommendation.

Another major factor in the diagnostic burden is the differentiation between malignant and benign disease. The current approach using an unenhanced CT-scan and if needed a wash-out CT-scan and FDG-PET scan is considered highly sensitive, but all modalities lack specificity (34-36). The low accuracy of malignancy risk assessment is underscored by studies demonstrating that more than one third of operated patients is eventually diagnosed with a nonfunctioning adenoma (30).
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Therefore, there is a need for alternative, simple and more accurate diagnostic tests. Urinary steroid profiling seems a promising tool for this purpose.

Retrospective studies demonstrated its high accuracy to discriminate benign from malignant adrenocortical tumors (37,38). In this thesis we demonstrated the influence of age and sex for steroid metabolite excretion in a healthy population. Whether deviation from normal values points towards relevant disease could not be delineated from this study. Incorporation of the identified influencing factors into an integrated analysis of steroid hormone metabolites might improve the diagnostic accuracy and should be further investigated. Furthermore, prospective validation of urinary steroid profiling in an adrenal incidentaloma population is needed before widespread implementation in clinical practice. One such initiative is the Systematic Evaluation of adRENaI tumors Discovered Incidentally – Prospectively Investigating the Testing Yield (SERENDIPITY) study that aims to include 1000 patients with an adrenal incidentaloma to determine the added value of a single urinary steroid profile compared to repeated CT-scans. Additionally, this study will be able to provide a comprehensive analysis of costs and quality of life associated with the adrenal incidentaloma work-up. Moreover, SERENDIPITY can provide a prospective validation of the accuracy of unenhanced CT-scanning to exclude a pheochromocytoma.

To conclude, the future challenge in the adrenal incidentaloma field is to develop and properly validate diagnostic tests that are accurate and simple to apply to a growing number of patients with a low pretest probability for clinically relevant disease.

Pretreatment of patients with an α-adrenergic receptor blocker before resection of a PPGL was first introduced around 1949 and has been part of clinical practice ever since (39). During surgical resection of a PPGL patients are at risk for a catecholamine crisis that can lead to hemodynamic instability, cardiovascular complications such as a cerebrovascular event or myocardial infarction, and ultimately death. The perioperative mortality rate has declined dramatically since the introduction of pretreatment from more than 25% to almost 0% nowadays (40-42). The cardiovascular complication rate is, however, still around 9% (42). Of note, it remains unknown whether the improvement in patient outcome is fully attributable to pretreatment because no placebo-controlled studies have been performed to date (43). Most likely, improvements of preoperative tumor
localization as well as anesthetic and surgical techniques also have contributed to the significant reduction in perioperative risk.

The Pheochromocytoma RandomisEd Study Comparing adRenoreceptor Inhibiting agents for Preoperative Treatment (PRESCRIPT) study was initiated to compare efficacy of different types of α-adrenergic receptor antagonists. No randomized controlled trial has ever been undertaken on this topic, most likely due to the many challenges associated with the rarity of this disease. The PRESCRIPT-trial focused on intraoperative hemodynamic variables as outcome measures. Both mortality and cardiovascular complications would have been superior endpoints, but are unrealistic considering the required number of patients in relation to the low incidence rate of PPGL. For example, more than 1000 patients would need to be studied to demonstrate a relative reduction of 50% in the number of cardiovascular complications. The relevance of the chosen study endpoints is underlined by previous extensive observations that demonstrated an association between hemodynamic variables and adverse postoperative outcomes in various patient populations that underwent general anesthesia (44-50). Moreover, almost every patient experiences blood pressure fluctuations during PPGL resection (51). We found that the cumulative duration outside the predefined intraoperative blood pressure targets, the primary endpoint of the trial, was not different between phenoxybenzamine and doxazosin. A favorable effect of phenoxybenzamine on the duration of hypertension was obscured in the composite primary endpoint due to the relative large contribution of hypotension. In agreement with this, analysis of the secondary endpoints demonstrated less severe hypertension and a lower requirement of vasodilating drugs in patients pretreated with phenoxybenzamine.

It should be noted that many variables can be assessed as a marker of hemodynamic instability, but individually they do not capture the full picture. Moreover, many of these variables interact with each other which complicates a straightforward analysis. For example, if a patient becomes hypotensive and is, subsequently, administered a vasopressive drug, then the blood pressure is likely to normalize. However, a normal blood pressure with the support of vasoactive drugs does not represent a physiologically normal hemodynamic state. Although a frequently used term (i.e. >13,000 PubMed citations) the definition of hemodynamic (in)stability is not used in a uniform matter (52). In an effort to assess the overall degree of hemodynamic instability we developed the hemodynamic instability score (HI-score) in which many relevant variables are integrated into a single clinical score. The methodology we used for the development is straightforward and the
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population was representative. The validation of this score is, however, complicated by the lack of a gold standard for hemodynamic instability. The added value of this score should be determined in future studies evaluating the relationship with, for example, postoperative adverse events or mortality in independent cohorts of patients that underwent general anesthesia. The application of the HI-score can theoretically be extended to any situation in which hemodynamic instability occurs, such as intensive care patients or post-anesthesia patients. Separate validation for these applications can be recommended. Commonly used cardiovascular drugs may vary between different patient populations and this should be taken into account when applying the HI-score. If necessary the point distribution of cardiovascular drugs should be modified. Of note, we also applied the HI-score to the PRESCRIPT-trial, after incorporating vasodilating drugs. We found an increased HI-score in patients who suffered from a postoperative cardiovascular complication. This observation should be regarded as the first external validation of the HI-score. In addition, HI-scores were lower in patients pretreated with phenoxybenzamine in accordance with other secondary outcome measures.

Taken all together, the PRESCRIPT-trial should be considered an inconclusive study on the primary endpoint, but secondary endpoints clearly suggest an advantage for phenoxybenzamine. We propose that this drug should be preferably used in the pretreatment of patients with PPGL. Limited availability due the narrow indication of phenoxybenzamine and associated high (unreimbursed) costs may hamper its use in daily clinical practice. Some authors even question the necessity for pretreatment altogether (43,53). It should, however, be realized that the data that is presented against pretreatment is heavily biased. For example, it is unclear how it was decided whether a patient would receive pretreatment, a large proportion of “non-pretreated” patients did receive other antihypertensive agents than α-receptor antagonists, details of intraoperative blood pressure deviations were poorly reported and no meaningful data on the amount of vasoactive drugs were provided (53). Moreover, the absence of a difference in complication rate lacks sufficient power for a meaningful interpretation. Furthermore, a longer duration of systolic blood pressure <80 and >200 mmHg has been demonstrated in untreated patients (54). However, it might be possible that not all patients benefit from pretreatment. An individualized approach to pretreatment should be substantiated by a reliable risk assessment that identifies patients with a low or high risk of hemodynamic instability. In an effort to delineate contributing factors we were able to explain only 15% of the variance in intraoperative hemodynamic instability by means of an exploratory analysis of the PRESCRIPT data. This
underlines the unpredictable hemodynamic consequences of PPGL and challenges the implementation of individualized pretreatment. Other potential influencing factors should be studied further to improve our understanding of the mechanisms that drive hemodynamic instability in patients with PPGL. For example, differences in catecholamine metabolism and secretion, single nucleotide polymorphisms that affect α-receptor activity, and downregulation of α-receptors might influence hemodynamic instability. A biobank containing pheochromocytoma tissue and DNA-samples of PRESCRIPT participants provides several opportunities to address these fascinating research questions.

**Conclusion**

The data in this thesis help to improve our understanding of adrenal tumors with a focus on diagnostic strategies and patient management. Ongoing efforts to enhance the discrimination between clinically relevant adrenal tumors and those without clinical consequences are supported by the development of an alternative diagnostic strategy. In addition, the findings described in this thesis should further optimize perioperative management of patients with PPGL, and may help to pave the way for a more comprehensive assessment of hemodynamic instability.
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References


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