CLINICAL PROTOCOL

A multicenter prospective study of biochemical profiles of monoamine-producing tumors: utility for diagnosis and determinants of clinical presentation

(The PMT-study: Prospective Monoamine-Producing Tumor study)

Study Leader *(Principal Investigator)*:

Prof. Dr. Stefan Bornstein

(Ärztliche Studienleitung, Dresden)

Medical Clinic III, Carl Gustav Carus University Clinic Dresden,

Fetscherstr. 74, 01307 Dresden

Datum des revidierten Prüfplans: 12 July 2010

TABLE OF CONTENTS

INSTITUTIONS & ASSOCIATE INVESTIGATORS

1. Carl Gustav Carus University Clinic Dresden, Dresden, Germany

Prof. Graeme Eisenhofer, PhD (Wissenschaftliche Studienleitung, IKL& MKIII)
Prof. Dr. Lorenz Hofbauer (Chief: Division of Endocrinology and Bone Metabolism, MKIII)
Prof. Dr. Hans-Detlev Saeger (Chair: Department of Surgery)
Prof. Dr. Jörg Kotzerke (Chair: Department of Nuclear Medicine)
Prof. Dr. Michael Laniado (Chair: Institut und Poliklinik für Radiologische Diagnostik)
Prof. Dr. Gustavo Baretton (Chair: Department of Pathology)
Prof. Dr. Gabriele Siegert (Chair: Institute of Clin. Chem. & Laboratory Medicine)
Prof. Dr. Angela Hübner (Division of Paediatric Endocrinology and Diabetology)
Dr. Roland Därr (Department of Endocrinology, MKIII)
PD Dr. Jacques Rohayem (Medizinisch-Theoretisches Zentrum)
Prof. Dr. Jacques Lenders, (Affiliated Professor, MKIII)
Dr. med. Barbara Klink (Institute of Clinical Genetics, Medical Faculty Carl Gustav Carus)

2. Radboud University Nijmegen Medical Center, Department of Internal Medicine and Department of Endocrinology, Nijmegen, The Netherlands

Prof. Dr. Jacques Lenders, (Department of Internal Medicine)
Dr. Henri Timmers (Department of Endocrinology)
Prof. Dr. Ad Hermus (Department of Endocrinology)

3. Institute of Cardiology, Department of Hypertension, Alpejska 42, 04-628 Warsaw, Poland

Prof. Dr. Andrzej Januszewicz (Department of Hypertension)
Dr. Aleksander Prejbisz (Department of Hypertension)

SYNOPSIS

Sponsor	None
Titel	A multicenter prospective study of biochemical profiles of monoamine-producing tumors: utility for diagnosis and determinants of clinical presentation
Kurzbezeichnung	The PMT-study: Prospective Monoamine-producing Tumor study
Zielpopulation (oder Indikation)	Patients with suspected monoamine-producing tumors: 1. Pheochromocytomas and Paragangliomas (PPGLs) 2. Gastroenteropancreatic tumors (GEPs)
Studiendesign	International multicenter prospective cohort study
Ziele der klinischen Prüfung	**Primary objective:** to identify new and improved disease biomarkers and establish the biochemical and molecular basis for variations in the clinical presentation of the different groups of tumors. **Secondary objectives:** • compare the diagnostic utility of urinary and plasma free metanephrines and establish an effective strategy for distinguishing true- from false-positive test results in patients with suspected PPGL. • to establish relationships between biochemical and metabolic profiles, underlying genotypes and presentation of disease. • to establish new and improved diagnostic biomarkers for GEP tumors and to compare their diagnostic utility with the currently available routine biochemical tests for these tumors. • to compare miRNA-profiles in patients with suspected and proven PPGLs or GEP tumors with healthy individual controls
Zielgrößen der klinischen Prüfung	• Numbers of true-positive, false-positive, true-negative and false-negative results of each biochemical test. • Biochemical, metabolic, molecular and secretatory profiles as related to the clinical manifestations, malignant development and germline mutations. • Blood pressure profiles, cardiac function parameters and indices of disease progression.
Patientenzahl	• patients with suspected PPGLs (total from all centers over 3 years n=2,400: an estimated 200 with PPGLs and 2,200 with tumors excluded; (Dresden 50 & 600; Nijmegen 50 & 600; Warsaw 100 & 1000). • patients with suspected or established GEPs (n=150: an estimated 50 with and 100 without GEPs). • healthy normotensive volunteers and primary hypertensive patients Total from all centers over 3 years n=300. (n=150 normotensives;150 hypertensives): for establishing reference intervals. (Dresden 100 & 50; Nijmegen 50 & 100).
Zeitplan	The study will last for five years but all patients will be enrolled in the first three years of the study. Follow-up will take place over minimum of two and a maximum of five years. • start date: 1-8-2010 • end date: 1-8-2015
Einschluss-kriterien	• male and female patients (all ages, including children above 5 yr) with suspected PPGLs if they fulfill one or more of the are following criteria: (i) patients with a previous history of PPGLs. (ii) new onset of hypertension or therapy-resistant hypertension or

	hypertensive episodes and/or symptoms suggestive of PPGLs. (iii) family history of PPGL or genetic mutations known to predispose individuals to develop PPGLs. (iv) presence of an accidently found adrenal tumor (v) any other reasonable clinical suspicion of a PPGL • adult male and female patients (all ages above 18 yr) with suspected GEP tumors based on clinical signs and/or symptoms. • for establishing reference intervals the following patients will be enrolled: (i) treated or untreated male and female patients with primary hypertension (>140/90 mm Hg) (above 18 yr). (ii) healthy normotensive volunteers (above 18 yr) All subjects must have read, understood and signed the informed consent form, before inclusion into the study protocol. Signed parental consent must be obtained for children with suspected PPGLs who are enrolled in the protocol and for healthy children who participate in the reference group.
Ausschluss-kriterien	• Patients with impaired mental capacity that precludes informed consent. • Subjects who need medications that may interfere with or invalidate outcome parameters (e.g., tricyclic antidepressants). • Pregnancy does not constitute criteria for exclusion from the protocol. However, in pregnant women no PET scanning, MIBG scanning or contrast CT will be performed. • Patients at risk from injury from the MRI magnet due to implantable metal or who suffer from anxiety in enclosed spaces are excluded from MRI.
Ablauf der klinischen Prüfung	All patients with suspected PPGLs will follow several diagnostic and follow-up phases as outlined in the table and in the flow chart. The first 3 phases are scheduled within the first 3 years of the study while the last phase will be carried out over the entire 5-year period. Since these patients will be seen in regular care, there is no fixed date schedule. Phase 1: patients enter the study for diagnostic testing. Phase 2: involves follow-up testing for confirmation of the biochemical diagnosis of pheochromocytoma in a subset of patients with equivocal test results. Phase 3: involves disease characterisation (only patients with biochemically confirmed PPGL). Phase 4: involves disease verification and patient follow-up. All patients with suspected GEP tumors will only follow phase 1 and phase 4 as outlined for PPGL. In all hypertensive and normotensive subjects, only blood and urine sampling will be carried out for establishing reference intervals for biochemical tests.

VISIT PLAN (for Patients with Suspected and Confirmed PPGLs)

	Phase 1	Phase 2	Phase 3	Phase 4
	Diagnostic screening	Biochemical confirmation	Disease characterisation	Disease verification and follow-up
Inclusion/exclusion criteria	X			
Informed consent	X			
Demographic data	X			
Medical history	X			
Physical examination	X			
EKG	X			X[7]
Plasma free metanephrines	X			X[7]
24-hr Urinary fractionated (deconjugated) metanephrines	X			X[7]
24-hr Urinary free metanephrines	X			X[7]
Serum/blood miRNA-profiles	X			X
Clonidine suppression test		X[1]		
Urinary free metanephrines (overnight sample)		X[1]		
24-hr Urinary catecholamines		X[2]	X[2]	X[7]
Plasma chromogranin-A		X[2]	X[2]	X[7]
Plasma catecholamines		X[2]	X[2]	X[7]
24-hr ambulatory BP measurement			X[3]	X[7]
Echocardiography			X[3]	X[7]
Pregnancy test[a]			X[3]	
CT or MRI			X[3]	
^{123}I-MIBG			X[3]	
^{18}FDG PET/CT				X[4]
Pathology of surgical specimen				X[5]
Genetic tests				X[6]

[1] Restricted to patients with equivocal (slightly elevated test results for plasma metanephrines or urinary fractionated metanephrines)
[2] Carried out only once under the protocol in either Phase 2 or 3 (i.e., not in both phases)
[3] Restricted to patients in whom the biochemical diagnosis of PPGL is confirmed
[4] Restricted to patients with inoperable PPGLs (e.g., metastases) for disease confirmation
[5] Restricted to patients with surgically resected tumors
[6] Only offered to patients with confirmed PPGLs
[7] To be carried out as part of post-operative follow-up in patients with confirmed PPGLs or as part of follow-up in patients with inoperable PPGLs
[a] If positive these female subjects will not have CT scan or ^{123}I-MIBG, but only MRI under the protocol

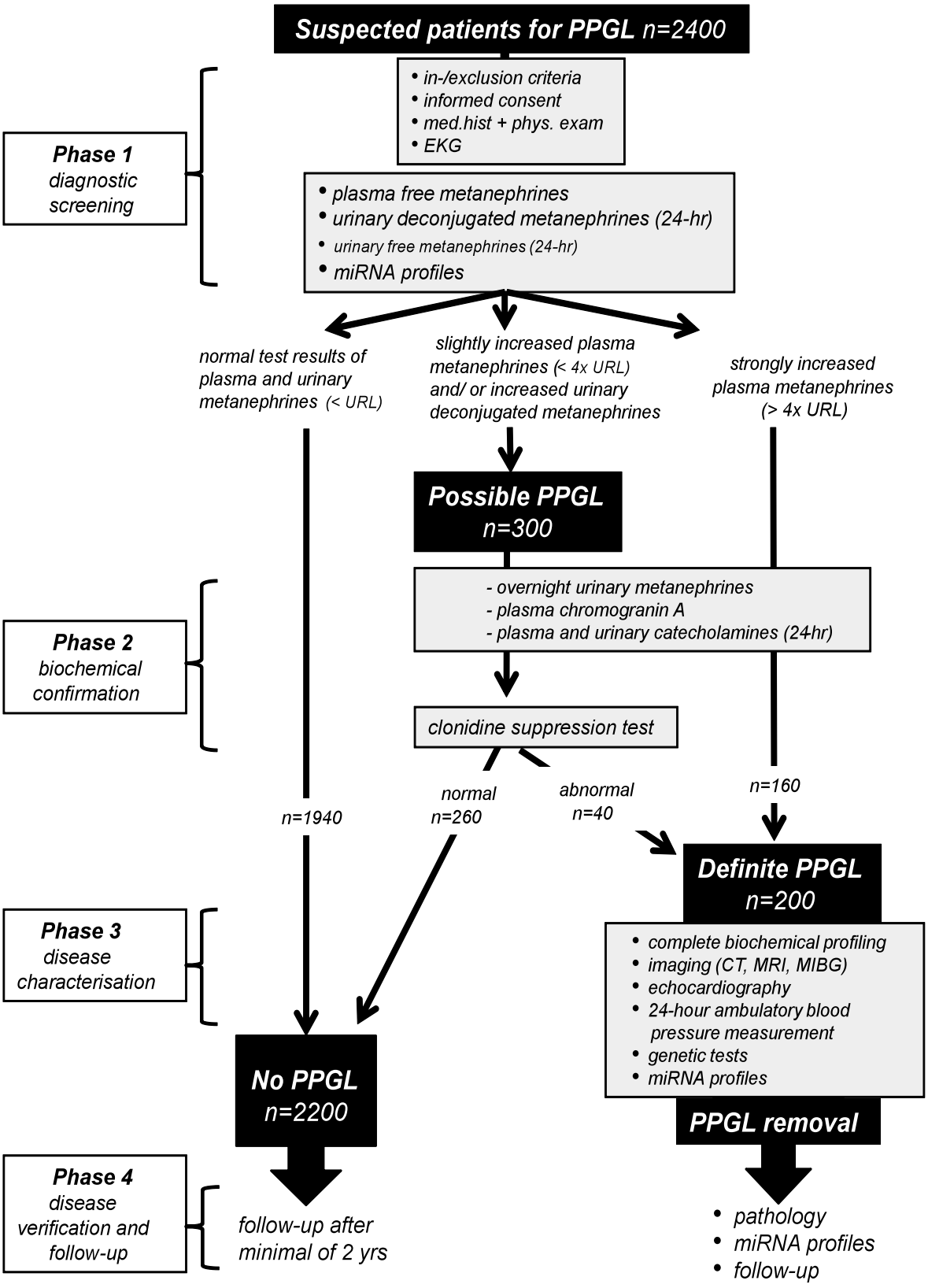

Figure 1. Flow chart for patients with PPGLs illustrating the four phases of the study, the summarized procedures for each phase and the numbers of patients expected to participate through each phase.

1. INTRODUCTION

1.1 BACKGROUND

Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumors that derive from adrenomedullary tissue (pheochromocytomas) in about 85% of cases and from extra-adrenal chromaffin tissue (paragangliomas) in 15% of cases (1). Carcinoids are tumors of enterochromaffin cells that belong to a heterogeneous group of neuroendocrine tumors, termed gastroenteropancreatic (GEP) tumors (2). Both groups of PPGLs and GEP tumors share similarities in that they are comprised of cells capable of amine precursor uptake and decarboxylation. They are also characterized by the presence of electron-dense chromogranin A (CgA) containing secretory vesicles, providing the basis for measurements CgA in plasma and tumor tissue as a biomarker for the tumors. Both groups of tumors also produce a range of monoamines; however, while PPGLs are characterized by production of mainly catecholamines, GEP tumors and in particular carcinoids more usually produce serotonin as the principle monoamine. These tumors consequently have quite different, but in some cases overlapping clinical presentations.

PPGL‘s represent a surgically correctable cause of hypertension, a result of their capacity to secrete catecholamines. As a consequence of the unpredictable, often explosive nature of this secretion, the tumors are potentially lethal if not quickly diagnosed and treated. Fortunately, once suspected, appropriate biochemical testing now makes it unlikely that the presence of a catecholamine-producing tumor will be missed. In particular, recognition that the O-methylated metabolites of catecholamines – the metanephrines – are produced continuously within chromaffin tumor cells and independently of variations in secretory activity has led to promulgation of these analytes as superior for diagnosis of PPGLs compared to other analytes (3, 4).

The nature of the production of metanephrines within tumor cells, means that these O-methylated metabolites are not only better diagnostic markers, they also provide a better reflection of the neurochemical phenotype of the tumors than the parent catecholamines (5, 6). Thus, the relative amounts of normetanephine to metanephrine measured in plasma or urine better indicate the relative amounts of noradrenaline and adrenaline in tumor tissue than measurements of the respective precursor catecholamines (7). Similarly, measurements of plasma methoxytyramine, the O-methylated metabolite of dopamine, provide a useful biomarker of tumor dopamine production (8). In contrast, urinary dopamine is derived almost exclusively from renal extraction and decarboxylation of circulating L-DOPA and does not provide useful information about tumor dopamine production.

PPGL‘s occur either sporadically or as a part of several hereditary syndromes. To date eight genes have been identified to be responsible for hereditary forms of these tumors. Mutations of the ret gene in multiple endocrine neoplasia type 2 (MEN2), of the von Hippel-Lindau (VHL) gene in VHL syndrome and of the neurofibromatosis type 1 (NF 1) in von Recklinghausen's disease are the most well known causes of hereditary PPGLs. Mutations of the genes encoding the 4 subunits of succinate

dehydrogenase (SDHA, SDHB, SDHC and SDHD) and of the succinate dehydrogenase complex assembly factor 2 (SDHAF2) represent more recently recognized causes of hereditary PPGL's. Most recently mutations of the gene for the transmembrane protein 127 (TMEM127) have been described as a further cause of hereditary PPGLs.

The clinical presentation and manifestations of PPGL's are highly variable and only now becoming understood to be influenced by the underlying mutation. Such influences include mutation-dependent effects on tumor location, propensity for malignancy, types of catecholamines produced, nature of catecholamine secretion, and resulting signs and symptoms (9-11). Tumors in MEN2 patients produce adrenaline leading to increases in plasma metanephrine, whereas those in VHL patients produce almost exclusively noradrenaline and are therefore characterized by increases of only plasma normetanephrine (12). Mutations of NF1, SDHD and SDHB genes also appear to lead to tumors with distinct catecholamine biochemical phenotypes (13). The phenotype associated with SDHB mutations often involves excessive production of dopamine and its metabolite methoxytyramine (14). Methoxytyramine — currently only measured in a few laboratories worldwide (including the Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Dresden) — appears to be a very useful marker of excess dopamine production, which in turn appears associated with malignancy (15). Interestingly, patients with mutations of SDHB genes are particularly prone to develop malignant tumors (9, 10, 16). Apart from this, there is currently no method to predict malignant potential or even diagnose the presence of malignant disease from immunopathological examination of a resected tumor. This diagnosis continues to depend on identification of metastases, at which stage the response to available therapies is limited.

Tumorigenesis in some of the hereditary PPGL syndromes, but particularly in malignant PPGL, appears to involve activation of hypoxia-angiogenic pathways linked to derangements in mitochondrial energy pathways. Mutations of succinate dehydrogenase related genes in particular, with subsequent block of the Krebs cycle, have fueled renewed interest in the Warburg effect in tumorigenic mechanisms. Among the hypotheses put forward to account for the link between the mutations of Krebs cycle enzymes and tumor development, accumulating evidence now strongly favors a mechanism involving inhibition of HIF prolyl hydroxylases by elevated levels of the Krebs cycle intermediate, succinate (17, 18). The resulting stabilization of HIF and the state of pseudohypoxia then leads to tumorigenesis and upregulation of glucose transporters and glycolytic enzymes, similar to the sequence in tumors due to mutations of the von Hippel-Lindau (VHL) gene. HIF also mediates reciprocal down-regulation of mitochondrial function and oxidative phosphorylation through several additional mechanisms (18). Such reciprocal relationships in the controls linking energy production by oxidative phosphorylation to that by anaerobic glycolysis allow new insight into how defects in a wide variety of oncogenes and tumor suppressor genes might lead to a common shift in energy metabolism favoring glycolysis over oxidative phosphorylation (19, 20). The shift to glycolysis drives an increased demand for glucose, explaining the exceptional utility of 18F-fluorodeoxyglucose (FDG) as a positron emission tomographic (PET) cancer-imaging agent. PPGLs with high uptake of 2-deoxyglucose

typically have more aggressive phenotype than those with lower FDG uptake (21). This observation supports other findings that a high glycolytic rate confers a proliferative advantage and must be a crucial component of the malignant phenotype (22). Reliance on glycolysis has been referred to as the metabolic Achilles heel of the cancer cell (23).

Excluding patients with malignant tumors due to mutations of SDHB genes, the otherwise relatively low rate of malignancy in patients with PPGLs contrasts with a much higher rate of malignancy in patients with GEP tumors. Also in contrast to PPGLs, GEP tumors even when suspected are often difficult to diagnose using currently available biochemical tests. Carcinoids most frequently produce serotonin, leading in about 10% of cases to the classic carcinoid syndrome. However, these tumors can also produce an array of other biogenic amines (e.g., histamine, dopamine, noradrenaline) and a variety of peptide hormones (2, 24). Additionally, some tumors are non-functional with no evidence of secretory products (particularly hindgut tumors). Others only produce monoamine precursors (particularly foregut tumors), reflecting deficiency of aromatic amino acid decarboxylase, the enzyme that converts 5-hydroxytryptophan to serotonin and L-dihydroxyphenylalanine to dopamine (25). Measurements of plasma CgA provides an alternative broadly applicable test; however, the test is non-specific and levels of CgA can be elevated into the pathologic range by drugs (e.g., proton pump inhibitors) and impaired renal function. Additional sub-optimal diagnostic sensitivity (46% to 73%), particularly for tumors with intermittent or little secretory activity, represents another critical limitation to reliance on serum CgA as a diagnostic test (26). Thus, currently there is no biochemical test that can reliably establish or exclude the diagnosis in all cases of GEP tumors (27, 28). The extremely varied and obscure clinical manifestations of these tumors likely reflects production of diverse secretory products, and presumably also contributes to confusion and delay in achieving a correct diagnosis (2).

Based on this extensive knowledge on the pathophysiology, diagnosis and treatment of PPGLs and GEP tumors, implementation of innovative approaches aimed at increasing the positive and negative predictive value of early stage diagnostics as well as post-therapeutic monitoring is appealing. One of those approaches is the so-called “miRNA-profiling”. MiRNA-profiling relies on the detection and monitoring in body fluids of non-coding RNAs, the so-called micro-RNAs (miRNA). Since the discovery of RNA interference (41), miRNAs have been recognized as regulators in numerous developmental and physiological processes. MiRNAs have been linked to cancer development and progression (42) as well as other diseases such as diabetes (43) and amyotrophic lateral sclerosis (44). A mammalian miRNA expression atlas based on small RNA library sequencing has been published by the NIH (45). Interestingly, it has been shown that HIF may be involved in up-regulation of miR-210 and miR-373, possibly implicated in DNA repair pathways (46). Recently, miRNAs in serum and blood are emerging as a new and promising class of biomarkers for the diagnosis of cancer and other diseases (47, 48).). In this context, a further aim of this project would be to characterize additional miRNA-biomarkers for PPGL and GEP through comparison in a prospective setting of the miRNA-profiles detected in serum and urine of the patients with the clinical patterns of disease and the biochemical profiles of the tumors. Moreovoer, miRNAs could also represent future therapeutic targets.

1.2 NECESSITY/RATIONALE OF THE STUDY

Although measurements of metanephrines in plasma or urine are now well established to provide diagnostic advantages over other analytes (e.g., catecholamines, vanillylmandelic acid and CgA) there remain considerable differences of opinion about the relative value and advantages of the different diagnostic measurements of metanephrines. These catecholamine metabolites may be measured in plasma or urine in either the free form or after a deconjugation step; in the latter case, measurements reflect the sum of free and sulfate conjugated metabolites. While measurements of free metanephrines in plasma may offer some advantages over measurements of fractionated deconjugated metanephrines in urine and plasma, such advantages have not yet been clearly established. Moreover, the diagnostic utility of measurements of urinary free metanephrines has yet to be formally studied, particularly in comparison to more commonly measured plasma free or urinary deconjugated metanephrines. The comparative utility of the various measurements of urinary and plasma free or deconjugated metanephrines for diagnosis of PPGL's therefore requires appropriate assessment, ideally by a multicenter prospective study involving large numbers of patients.

While measurements of plasma and urinary metanephrines offer high diagnostic sensitivity, specificity remains suboptimal. Due to this and the extremely low pretest prevalence of PPGLs among tested patients, false-positive results remain a major problem. Several strategies have been suggested to differentiate true- from false-positive results, including follow-up measurements of urine catecholamines and CgA and use of clonidine suppression testing (29, 30). However, all such studies to date on these strategies have been retrospective. Additionally most studies have not included appropriate reference groups; more importantly, deficient gold standard methods have usually been used for exclusion of the tumor. Because of all these drawbacks, a prospective multicenter study is also warranted to establish an effective and efficient algorithm for follow-up testing in patients with positive test results and in whom there remains a need to further rule out or prove the tumor.

Recognition that germ-line mutations of tumor susceptibility genes are responsible for at least 30% of all PPGL's, including those where there is no obvious hereditary basis, provides an argument for genetic testing in all patients with paraganglial tumors (31). However, at an international meeting convened to develop guidelines on this and other issues, it was recommended that "although there is now a reasonable argument for more widespread genetic testing, it is not currently cost-effective to test every disease-causing gene in every patient with a pheochromocytoma or paraganglioma (4, 32). Rather, the decision to test and which genes to test require judicious consideration of numerous factors." The catecholamine biochemical phenotype was listed as one factor potentially useful to guide decision-making. Preliminary data also suggest that the catecholamine biochemical phenotype may be additionally useful for assessing future potential or presence of malignant disease in patients with PPGL's. Whether such neurochemical information may be used for predicting malignant disease or the presence of specific underlying mutations (and therefore as a guide to genetic testing) is again best assessed in the setting of a prospective multicenter study of PPGL's.

Phenotypic differences in hereditary PPGL's due to differences in biochemical and catecholamine secretory phenotypes may also explain extreme variability of clinical presentation in patients with more common sporadic tumors. For example, diabetes and insulin resistance are common features of patients with PPGL's, with preliminary evidence suggesting an association with adrenaline-producing tumors. Highly variable cardiovascular manifestations — including paroxysmal versus sustained hypertension, stroke, hypertrophic cardiomyopathy, shock and multiple organ failure — likely also reflect differences in catecholamine secretory profiles and underlying differences in the expression of secretory pathway components. Such relationships are important to assess not only for understanding and predicting morbidity associated with PPGLs, but also for gaining insight into potential mechanisms of more common clinical disorders involving a pathogenic link to sympathoneural and adrenal release of catecholamines (e.g., essential hypertension, Tako Tsubo cardiomyopathy).

The nature of the underlying mutations in hereditary PPGL's make these tumors especially appropriate for investigation of the Warburg effect. In particular, the heterogeneous nature of the tumor phenotypes associated with SDH subunit mutations raise critical questions that once answered may shed light on the malignant transformation process. Improved understanding of how altered energy metabolism might contribute to progression of cancer is now leading to consideration of new therapeutic approaches aimed at small molecule energy pathways (33, 34). Availability of clinical material through this protocol will enable hypotheses about small molecule energy pathways to be addressed, which in combination with bench level studies employing model systems should lead to improved understanding of tumorigenic processes and identification of potential therapeutic targets.

Although the incidence of GEP tumors is as much as 10 times higher than that of PPGL's, the development of new and effective diagnostic tests for the former tumors has lagged far behind that for the latter tumors. Consequently the biochemical diagnosis of carcinoids remains difficult. Compounding the problem is the highly diverse clinical presentation and more often malignant nature of these tumors compared to PPGL's. There is thus not only a need for new and improved tests to diagnose the tumors at an earlier stage in their development, but also tests that offer improved utility as surrogate markers for therapeutic monitoring.

There is no specific miRNA pattern known to be associated with either related to GEPs or to PPGLs, but possibly patients with GEPs or PPGLs may be distinguished from those without tumors by characteristic miRNA-patterns. This protocol will also offer the opportunity to compare biochemical profiles with miRNA patterns and establish other possible differences according to tumor location and size, presence of metastases and other features associated with clinical presentation of the tumors (e.g., hypertension). Moreover, because to date miRNA-profiling typically relies on a cost-intensive and time-consuming technology (i.e., microarray genotyping), such studies are rather cumbersome and require extensive human and material resources. A new and innvotive technology has been developed at Riboxx Inc. (Riboxx GmbH, Germany) that allows easy and straight-forward detection of miRNA in

body fluids. This technology is based on RNA-Synthesis by the RNA-dependent RNA-polymerase (RdRp) of the Calicivirus. The RdRp opf the calicivirus initiates RNA-synthesis in a primer-independent manner on RNA-templates, allowing the amplification of miRNA that are per se of 15-35 nt in length from clinical samples. The Riboxx-technology will be implemented in this project in order to allow quantitative real-time detection of miRNAs of interest in vitro. Detection occurs in a High-Throughput format (384-Well-fomat) in a sensitive and specific FRET-based system. Using the Riboxx-Technology, a detection of about 1000 different and well characterized miRNAs will be performed. The miRNA-based approach will allow the genotyping profiling of the tumors and further correlation of genotype to clinical phenotype.

2. OBJECTIVES OF THE STUDY

2.1 PRIMARY OBJECTIVE

The **long-range goal** of the research planned under this protocol is to develop new and improved approaches for diagnosis, management and treatment of patients with monoamine-producing tumors, including PPGLs and GEP tumors. As one of the steps towards attaining this goal, the **primary objective** of this study protocol is to identify new and improved disease biomarkers and establish the biochemical and molecular basis for variations in the clinical presentation of the various groups of tumors. A **central hypothesis** is that the varied course of clinical manifestations and complications of monoamine-producing tumors reflect underlying differences in biochemical phenotypes, which in turn depend on the specific tumor cell types and the underlying mutations responsible for the tumors. The **rationale** underlying this project is that elucidation of the relationships between biochemical phenotypes, genotypes and the natural history of the disease will lead to improved understanding of tumor biology and development of new and improved approaches for diagnosis, management and treatment of the tumors.

2.2 SPECIFIC AIMS

The observational cohort study covered by this protocol has the following **specific aims**:

1. Compare the utility of measurements of urinary free metanephrines, plasma free metanephrines and standard tests of urinary deconjugated metanephrines as initial tests for diagnosis of catecholamine-producing PPGLs.

2. Prospectively establish an effective and efficient strategy for follow-up biochemical testing to distinguish true-positive from false-positive elevations of plasma or urinary metanephrines in patients with suspected catecholamine-producing PPGLs.

3. Characterize the biochemical signatures of different types of catecholamine-producing PPGLs and prospectively determine whether this information can be used to predict tumor burden, the

presence or subsequent occurrence of malignant disease and the relative likelihood of specific mutations as a cost-effective and efficient guide to genotyping.

4. Establish the relationships between the molecular, biochemical and secretory phenotypes and the cardiovascular and metabolic complications of catecholamine-producing PPGLs.

5. Utilize mass spectrophotometric (MS) based metabolomic profiling to characterize the neurochemical profiles and bioenergetic signatures of different groups of hereditary PPGLs and any relationship of these profiles and signatures to tumor aggressiveness and malignancy.

6. Extend HPLC- and MS-based monoamine metabolomic profiling to GEP tumors (including carcinoids) with the aim in a pilot study of identifying a panel of novel tumor biomarkers that can be compared for diagnostic efficacy against currently available routine biochemical tests for these tumors (e.g., 24-hour urinary 5-HIAA and serum CgA).

7. Establish whether miRNA profiling of serum samples may provide novel biomarkers of disease in patients with PPGLs and GEP tumors. In particular, comparisons with well-established and validated biochemical tests offer the advantage of a “gold standard” for validation of the miRNA-profiles of the tumors.

3. INVESTIGATIONAL PLAN

3.1 OVERVIEW OF THE STUDY DESIGN AND PLAN

This is an international multicenter prospective cohort study of patients with suspected PPGLs. Patients also include a limited number of patients with suspected GEP tumors for an initial pilot study to identify novel tumour biomarkers that might be further explored for diagnostic efficacy in future studies. Healthy normotensive volunteers and patients with primary hypertension are also included for purposes of establishing reference intervals of biochemical tests and for other comparisons involving collections of biological specimens for metabolic and biochemical profiling. The study is planned for five years and involves 4 phases. The first phase, to be carried out during the first three years in all subjects recruited into the study, involves initial diagnostic testing (blood sampling and urine collections). The second phase, also restricted to the first three years of the study, involves follow-up diagnostic testing in a subset of patients with suspected PPGLs. The third phase, also to be carried out during the first 3 years of the study, involves disease characterization in a further subset of patients in whom biochemical testing in phases 1 and 2 establishes the presence of PPGLs. Phase 4 involves disease verification and patient follow-up to be carried out over the entire 5-year period in all patients with established or suspected PPGLs or GEP tumors who are recruited into the study.

3.2 PRIMARY OUTCOME PARAMETERS

The primary outcome parameters for the objectives related to evaluation of the 3 biochemical tests for initial diagnosis of PPGLS (specific aim 1) are estimates of diagnostic specificity and sensitivity, which will be combined into a single measure of diagnostic accuracy (see Section 3.9 - Power Analysis). Diagnostic sensitivity and specificity are determined from numbers of true-positive, false-negative, true negative and false-positive results for each biochemical test.

Patients in whom disease is confirmed provide the primary outcome parameters for true-positive and false-negative results. Patients in whom disease is excluded provide the primary outcome parameters for false-positive and true-negative results.

In addition to the actual biochemical test results, the primary outcome parameters require availability of established reference intervals and independent methods for diagnosis verification. The reference intervals are established from measurements of analytes in healthy normotensive volunteers and patients with essential hypertension and are used to establish whether biochemical test results are positive or negative. Diagnosis verification is by pathological examination of resected tumor tissue to establish the presence of disease and patient follow-up to exclude disease. However, in patients with malignant or inoperable tumors in whom resected or biopsied tumor material is unavailable, disease verification is by imaging evidence of metastases or an inoperable tumor, accompanied by previous independent biochemical evidence or a history of a previously resected tumor.

3.3 SECONDARY OUTCOME PARAMETERS

Secondary outcome parameters include the molecular, miRNA, biochemical, secretory and metabolic profiles as related to the presence of specific underlying germ-line mutations, presence or development of malignant disease, and the clinical manifestations of the PPGLs. Other secondary outcome parameters include blood pressure profiles, parameters of cardiac function, tumor size and location data derived from imaging procedures and other indices of disease progression.

3.4 PATIENTS AND SUBJECTS

(i) Patients with suspected PPGLs (total from all centers over 3 years n=2,400, includes an estimated 200 with PPGLs and 2,200 with tumors excluded; Dresden - 50 & 600; Nijmegen - 50 & 600; Warsaw - 100 & 1000.

(ii) Patients with suspected or established GEPs (total over 3 years n=150, includes an estimated 50 with and 100 without GEPs).

(iii) Healthy normotensive volunteers and primary hypertensive patients (total from Nijmegen and Dresden centers over 3 years n=300; includes 150 normotensives and 150 hypertensives) Dresden – 100 normotensives & 50 hypertensives; Nijmegen – 50 normotensives & 100 hypertensives.

3.5 INCLUSION CRITERIA

3.5.1 Patients with suspected PPGLs

Male and female patients (all ages, including children above 5 years) with suspected PPGLs are included on the basis of one or more of the following:

(i) Patients with a previous history of PPGLs.

(ii) New onset of hypertension or hypertensive episodes and/or symptoms suggestive of PPGLs (sweating, headache, pallor, palpitations, or other suspicious spells)

(iii) Therapy-resistant hypertension, defined as an office blood pressure of >140/90 mmHg despite treatment with >3 antihypertensive agents at full dose (including a diuretic).

(iv) Family history of PPGL or genetic mutations known to predispose individuals to develop PPGLs.

(v) Presence of an adrenal or retroperitoneal mass discovered incidentally during abdominal imaging studies carried out for investigations unrelated to clinical suspician of PPGLs.

(vi) Any other situation involving reasonable clinical suspicion of a PPGL (e.g., patients with a vasopressor response during anesthesia, surgery or angiography).

3.5.2 Patients with GEP tumors

Adult male and female patients (all ages above 18 yr) with proven or suspected GEP tumors are included on the basis of one or more of the following:

(i) Patients referred or recruited because established GEP tumors.

(ii) Patients suspected to have GEP tumors based on clinical signs and presenting features (e.g., flushing, diarrhea, steatorrhea, wheezing, dyspepsia, ulcers, hypoglyceamia, heart disease, deep vein thrombosis, anorexia, nausea, vomiting, constipation, hypotension, fainting, skin disorders, dumping syndrome, pernicious anaemia, autoimmune disorders, diabetes, gall bladder disease).

(ii) Patients suspected to have GEP tumors based on previous biochemical testing and/or imaging studies.

3.5.3 Primary hypertensive patients and healthy normotensive volunteers

Currently there are no reference levels established for urinary free metanephrines. All other reference intervals also require updating, particularly as diagnostic assays move from HPLC electrochemical detection based methods to liquid chromatography with tandem mass spectrometry based methods. Adult male and female healthy normotensive subjects and patients with primary hypertension (all ages above 18 yr) are therefore selected according to the below criteria:

(i) Treated or untreated patients with hypertension in whom a secondary cause of hypertension has been excluded according to the standard clinical criteria. Hypertension must be documented by systolic blood pressure over 140 mmHg and diastolic blood pressure of over 90 mmHg on three separate dates that blood pressure measurements were obtained

(ii) Healthy normotensive volunteers who are not under specialist treatment for any disorder or disease, who are taking no antihypertensive medications and who have a normal blood pressure of <140/90 mm Hg.

3.5.4 Antihypertensive medications

Patients taking antihypertensive medications are not excluded from the study. Since antihypertensive medications have minimal influences on the primary outcome parameters, these medications will not be withdrawn unless this is considered medically indicated. Where such medications are withdrawn, this will be carried out according to standard medical care (e.g., in taperied doses) and with consideration of appropriate replacement therapy as required. Examples include patients on beta-adrenergic blockers, in whom there can be a risk of hypertensive crises should there be a PPGL (35).

3.5.5 Children – justification for entry into the protocol

Among 361 patients enrolled into previous NIH/Nijmegen PPGL clinical protocols, 39 (10.8%) were aged 18 years or younger at the time of initial diagnosis of their tumors. Among that group, 33 (85%) had established mutations of tumor susceptibility genes. These data serve to emphasize the importance of including children in studies of PPGLs, particularly children identified at risk for the tumors due to inherited susceptibility. Similarly, the data also illustrate the importance of identifying at an early age patients at risk for PPGLs because of germ-line mutations of tumor susceptibility genes. Once such mutations are identified, patients should be screened for PPGLs at regular intervals (normally yearly) according to established standard of care clinical guidelines. Early detection of tumors can avoid potentially fatal complications and in children with SDHB mutations may be particularly important for minimizing risk of subsequent malignant disease.

The clonidine test, CT scans and fluororodeoxyglucose PET scans will not be offered to children as part of the protocol. These procedures may, however, be carried out as clinically indicated.

Children are only enrolled into the protocol with signed consent of their parents or legal guardians. The consenting process for children also includes witnessed verbal assent according to prepared scripts (see verbal assent forms for children 5 to 10 yrs and 11-18 years).

3.5.6 Signed informed consent

All adult subjects must have read, understood and signed the informed consent form, before inclusion into the study protocol. Signed parental consent must be obtained for children to be enrolled in the protocol. Children enrolled into the study must provide verbal assent that they agree to their participation and will be provided with the opportunity for signed consent if they can write and sign their own name.

3.6 EXCLUSION CRITERIA

(i) Subjects with impaired mental capacity that precludes informed consent.

(ii) Subjects who require medications that would interfere with or invalidate primary outcome parameters (e.g., tricyclic antidepressants).

(iii) Pregnant women will not be included as part of either normotensive or hypertsive control groups. Apart from this pregnancy or advanced age does not constitute criteria for exclusion from the protocol. However, pregnant women are excluded from receiving clonidine under the protocol and from all portions of the protocol involving administration of radioactivity. In women of childbearing age (up to age 50) a pregnancy test is performed. In those with a positive result, MRI will be used as an imaging modality but no PET scanning, MIBG scanning or contrast CT will be performed under the protocol.

(iv) Children will not be included as part of either normotensive or hypertensive control groups. Children below 5 years of age are also excluded from all portions of the protocol. Apart from the above, children aged 5 to 18 yrs are not excluded from the protocol. However, children are excluded from the clonidine test, computed tomography and ^{18}F-fluorodeoxyglucose PET under the protocol. These procedures may, however, be carried out when clinically indicated outside of the protocol.

(v) Patients at risk from injury from the MRI magnet due to implantable metal or who suffer from anxiety in enclosed spaces are excluded from parts of the study involving MRI.

3.7 RECRUITMENT OF PATIENTS AND NORMOTENSIVE VOLUNTEERS

3.7.1 Recruitment of patients of patients with PPGL and GEP tumors

Recruitment of patients with suspected PPGL or GEP tumors will be carried out continuously on a referral basis, through admissions and by follow-up of patients according to medical history or known hereditary condition. Additional patients will be recruited through off-site enrollment, as detailed further below.

3.7.2 Recruitment of normotensive volunteers and patients with primary hypertension

Normotensive volunteers and patients with primary hypertension will be recruited into the protocol by referral and advertisement.

3.8 STUDY PLAN AND METHODS

Patients will be examined and treated according to the existing and accepted standards of clinical care. The protocol includes no experimental interventions or treatments outside of those routinely used in clinical care. Importantly, the only additional procedure in patients with PPGLs or GEP tumors that might be considered to extend beyond routine clinical care is the taking of additional blood for miRNA

profiling and banking; however, this additional blood will only be taken at the time of blood draws for standard of care diagnostic testing; we consider this extra procedure justified as this may provide us with novel biomarkers for future sensitive diagnosis and precise therapy monitoring. All evaluations may be performed on an outpatient basis. However, in certain situations (distance to hospital, severity of symptoms etc) patients may be evaluated on an in-patient basis. Also the presurgical medical preparation can be partially or completely carried out on an in-patient basis. In healthy normotensive subjects procedures will be limited to histories and physicals and sampling of blood and urine. For the protocol, similarly limited procedures will also apply to patients with primary hypertension.

3.8.1 Procedures

(i) **History & Physical (H&P):** A standard H&P is carried out in all subjects enrolled into the protocol. In those with suspected monoamine-producing tumors there is additional emphasis on family history, the cardiovascular system (e.g., hypertension, hypotension, tachycardia, bradycardia, chest pain or pressure, palpitations, dyspnea, syncope, orthostatic intolerance, heart disease) and relevant signs and symptoms or other clinical manifestations of monoamine-producing tumors (headache, transient neurological deficits, tremor, convulsions, dizziness, altered sweating, pallor, acrocyanosis, anxiety, abdominal discomfort, flushing, diarrhea, steatorrhea, wheezing, dyspepsia, ulcers, hypoglyceamia or hyperglyceamia, deep vein thrombosis, anorexia, nausea, vomiting, constipation, skin disorders, dumping syndrome, pernicioius anaemia, autoimmune disorders, diabetes, gall bladder disease). Relevant clinical information is recorded using a standardized form.

(ii) **EKG:** To be carried out in all patients as part of Phase 1 with suspected monoamine-producing tumors. The test is primarily carried out as an addition to the history and physical once patients have been enrolled into the protocol; however, results of the test are also of use for addressing specific aim #4.

(iii) **Blood and urine sampling for biochemical diagnosis of PPGLs and GEP tumors:** All subjects with suspected PPGLs undergo diagnostic screening measurements of baseline levels of plasma free metanephrines and 24-hour urine collections of fractionated free and deconjugated metanephrines. Blood will be drawn from an indwelling cannula in the brachial vein after 30 minutes of supine rest. Patients are instructed to fast overnight and to refrain from alcohol and nicotine and caffeinated and decaffeinated beverages for 12 hours before sampling. They are not to take acetaminophen (Paracetomol) for 5 days before sampling. For patients with suspected GEP tumors, blood and urine for diagnostic testing will be collected under the standard procedures of collection for tests available at each center and with the appropriate drug and dietary restrictions. At all participating centers, additional blood (40 mL maximum) and urine is collected to allow banking of extra samples at -80^{o}C for additional neurochemical testing of monoamine and metabolomic profiling in patients once diagnoses are established (see also section 3.8.1 xviiI on specimen banking below). These collections of urine and blood samples are carried out under Phase 1. They are therefore of primary importance to address specific aims #1 and #6. The measurements in patients with suspected PPGLs, however, are also

essential to all other specific aims.

(iv) **Clonidine suppression tests:** Clonidine suppression tests are clinically indicated for assisting in the differential diagnosis of PPGLs and hypernoradrenergic states. In this protocol clonidine suppression testing will be limited to patients with suspected PPGLs in whom initial tests of plasma free metanephrines yield positive test results but are of insufficient magnitude to unequivocally establish the presence of a tumor (i.e., for such patients entering Phase 2). The drug is given orally at a dose of 300 micrograms (0.3 mg) for patients in the 65 to 75 kg weight range. For patients outside this weight range the dose is adjusted at 4.3 µg/kg. Blood pressure and heart rate are monitored and blood is drawn via an i.v. cannula for determination of catecholamines and metanephrines before and at 3 hours after clonidine administration. This dose of clonidine causes a slight fall in blood pressure in most patients with hypertension of any cause. Plasma levels of noradrenaline and normetanephrine fall in almost all patients in whom elevated levels are due to increased sympathetic activity (i.e., those patients with false-positive elevations of plasma normetanephrine for initial tests of plasma free metanephrines). However, in most patients with PPGLs, plasma levels of noradrenaline and normetanephrine do not show relevant falls after the drug. The test is of primary importance for addressing specific aim #2.

(v) **Overnight urine collections:** Overnight urine collections will be limited to patients with suspected PPGLs in whom initial tests of plasma free metanephrines show increases that are of insufficient magnitude to unequivocally establish the presence of a tumor (i.e., for such patients entering Phase 2). Overnight urine collections avoid potential increases in urinary catecholamines and catecholamine metabolites associated with increased sympathoadrenal activity during daily activities and have therefore been advocated as a useful method to minimize false-positive results (36). The test is of secondary importance for addressing specific aim #2.

(vi) **Twenty-four hr urinary catecholamines:** Urine collections for measurements of 24-hr outputs of urinary catecholamines will be carried out in patients with suspected PPGLs in whom initial tests of plasma or urinary metanephrines yield positive test results but are of insufficient magnitude to unequivocally establish the presence of a tumor (i.e., for such patients entering Phase 2). For those patients the measurements are of secondary importance for addressing specific aim #2. The measurements, however, are also important for disease characterization and for satisfying aims 3 and 4. Therefore, 24-hour urine collections for catecholamines are also carried out in patients where initial tests of plasma free or urinary metanephrines unequivocally establish the diagnosis of a PPGL (i.e., for patients bypassing Phase 2 and directly entering Phase 3).

(vii) **Plasma catecholamines and chromagranin A (CgA):** Blood collections for measurements of plasma catecholamines and CgA will be carried out in patients with suspected PPGLs in whom initial tests of plasma or urinary metanephrines yield positive test results but are of insufficient magnitude to unequivocally establish the presence of a tumor (i.e., for such patients entering Phase 2). For those patients the measurements are of secondary importance for addressing specific aim #2. The measurements, however, are also important for disease characterization and for satisfying aims 3 and 4. Therefore, blood collections for catecholamines and CgA are

also carried out in patients where initial tests of plasma free or urinary metanephrines unequivocally establish the diagnosis of a PPGL (i.e., for patients bypassing Phase 2 and directly entering Phase 3).

(viii) **24-Hour ambulatory blood pressure monitoring (ABPM):** To be carried out as part of Phase 3 in all patients with PPGLs. Patients with PPGLs will undergo non-invasive BP monitoring over a 24-hour period. The blood pressure monitoring equipment is lightweight, small and will barely restrict patient activity. The device consists of a blood pressure cuff, which is placed around an arm, and which self inflates and automatically measures blood pressure at programmed intervals, every 15 minutes during the day and every half hour at night. The patient is fitted with the device on one day, is then free to go with his/her daily activities. The following day, 24 hours or more after fitting, the patient returns to the clinic to have the device removed. The patient fills out a diary, to relate events and activities (e.g., eating, exercise, sleep, medications) to blood pressure during the 24 hours of monitoring. The data from this procedure are of primary importance for addressing specific aim #4.

(ix) **Echocardiography:** To be carried out as part of Phase 3 in all patients with PPGLs. Data obtained by this test include cardiac ejection fraction, diastolic left ventricle internal diameter, ventricle end-systolic volume, left ventricle end-diastolic volume, mitral valve E/A ratio and pulmonary vein S/D ratio. These data are of primary importance for addressing specific aim #4.

(x) **Pregnancy test:** To be carried out as part of Phase 3 in all female patients of child-bearing age with unequivocal biochemical evidence of PPGLs. The test is carried out to establish eligibility for the various imaging procedures carried out under the protocol as described below.

(xi) **Computed tomographic and magnetic resonance imaging:** The conventional imaging tests carried out under this protocol include either computed tomographic imaging or magnetic resonance imaging (head, neck, chest, abdomen, and pelvis). Such imaging procedures are routinely required for localization of biochemically-proven PPGLs. In this protocol, either or both of these imaging procedures are likewise carried out in all patients with unequivocal biochemical evidence of PPGLs as part of Phase 3. The data from these procedures are important for addressing specific aim #3. More specifically, predictions about tumor burden and location derived from biochemical signatures are verified using the data from imaging studies. In conjunction with the data from biochemical testing, the data from imaging studies are also essential for establishing cost-effective and efficient guide for genotyping.

(xii) **^{123}I-MIBG scintigraphy:** This nuclear imaging procedure is routinely carried out for diagnostic localization of PPGLs, usually once tumours are confirmed by biochemical testing and located by CT or MRI, but sometimes when tumors cannot be located by conventional imaging even when biochemical testing is not conclusive. However, for the purposes of the protocol ^{123}I-MIBG scintigraphy will be carried out only where biochemical evidence of PPGLs is clear under Phase 3. The imaging procedure is carried out as part of excepted standards of care to identify a mass as a PPGL, to locate masses that are not identified by conventional imaging and to establish absence or presence of multifocal or metastatic disease.

(xiii) **^{18}Fluorodeoxyglucose positron emission tomography (FDG-PET/CT):** This nuclear

medicine imaging procedures will be carried out under the protocol as part of disease verification and follow-up (Phase 4) in a small subgroup of patients with inoperable PPGLs associated with malignant disease or in patients with SDHB mutations, who have a high risk of malignant disease and in whom FDG-PET provides superior sensitivity over ^{123}I-MIBG for localization of metastases (37). Both FDG-PET and ^{123}I-MIBG imaging procedures are recommended for localization of metastases in patients with malignant PPGLs (4). For the purposes of the protocol, these imaging procedures also provide independent methods for disease verification in patients in who surgically resected tumor tissue is unavailable for histopathological confimation of disease. The data so obtained are essential for satisfying specific aims #1 through #5.

(xiv) **Genetic testing:** To be offered on a pseudonymized basis to all patients with definitive evidence of PPGLs, either as part of Phase 3 or 4. At least 30% of all patients with PPGLs, including up to 24% who initially present with sporadic PPGL, have mutations of *RET*, *VHL*, *SDHB and SDHD* genes (31). Including those also with PPGLs due to mutations of the NF1 gene and the more recently described SDHAF2 and TMEM127 genes, the proportion of hereditary PPGLs is likely to exceed 30% and possibly reach 40%. Patients with most of these mutations carry a high risk of PPGLs and other tumors depending on the particular mutation (e.g., medullary thyroid cancer in patients with RET mutations). The prognosis of patients with such mutations is considerably improved by their enrollment into periodic surveillance plans. Such plans allow earlier detection and treatment of tumors that may otherwise have a more unfavorable outcome. Consequently it is recommended that genetic testing should be considered for all patients with PPGLs (4). In keeping with this recommendation, genetic testing will be offered to all patients with proven PPGLs. Patients will be first counseled on the ramifications of a positive result (i.e., an identified mutation), including implications for other family members and how in some nations such results may be considered a pre-existing medical condition with unfavorable consequences for eligibility for health insurance. It has been the experience of the study leaders of this protocol that patients with PPGLs rarely refuse the invitation to have their DNA screened for mutations of cancer-causing genes once the health implications of an unrecognized mutation have been explained. For the purpose of genetic testing, 10 ml of whole blood is collected in EDTA or heparin-coated tubes to prevent clotting. Samples for DNA testing are collected and stored pseudonymized in coded tubes without any other attached information that would allow identification of the individual from whom the sample is collected. Genes to be tested include exons 10, 11, 13 and 16 of the RET proto-oncogene and all exons of VHL (exons 1-3), SDHB (exons 1-8), SDHD (exons 1-4) and TMEM127 (exons 1-4) genes. Large deletions of SDHD, SDHB and TMEM127 genes will additionally be evaluated by quantitative multiplex PCR of short fluorescent fragments (QMPSF). Multiplex-ligation-dependent probe amplification (MLPA) is carried out for the VHL genes. These procedures may be adapted according to future technological advances. If a mutation is detected, the patient receives further counseling on how this may impact his/her future health and the surveillance plans recommended for that particular mutation. Genetic testing is also offered to at risk family members. The results of genetic testing are essential for categorizing patients into groups with sporadic and familial PPGLs, and within the latter category

for defining the particular mutation. These data are essential for addressing specific aim #3, and specifically addressing how biochemical profiles may be combined with other clinical information (e.g., tumor location, patient age) to establish cost-effective and efficient guidlines for genetic testing. The data from genetic testing are also essential for addressing specific aims #4 and #5.

(xv) **Pathology:** Histopathological examinations are carried out on all resected tumor specimens as part of Phase 4 for purposes of disease verification. Verification of disease by an experienced and qualified pathologist is essential for addressing all specific aims of the protocol. Several specialized immunohistochemical and scoring procedures will also be carried out to assist in addressing specific aims 3 and 5. Initial evaluation of PPGLs is made under the light microscope in haematoxylin and eosin stained sections. Findings of distinctive polygonal cells with amphophilic or basophilic cytoplasm and small, spherical or ovoid, pale-staining nuclei are typical of PPGLs. Cytological features can, however, often be non-descript. For such cases in which the diagnosis is uncertain, differential diagnosis is based primarily on immunohistochemical examination for expression of chromogranin A, which distinguishes PPGLs and GEP tumors from tumors that are not neuroendocrine, such as those derived from the adrenal cortex (38). Staining for keratins (usually absent or minimal in PPGLs) and tyrosine hydroxylase (usually positive in PPGLs) are usually sufficient to distinguish catecholamine-producing PPGLs from other neuroendocrine tumors, including GEP tumors (38). S-100 protein provides another useful marker with intense nuclear staining providing identification of PPGLs, including both sympathetic and parasympathetic paragangliomas. Immunohistochemical stainings will include SDHB, which has been recently introduced to discriminate patients with mutations of SDH genes from those with other mutaions (39). It is not possible using histopathological methods to distinguish malignant from benign PPGLs. However, several scoring systems have been introduced in an attempt to assist in this distinction, including the PASS score described by Thompson in 2002 (40). This system has been adapted and is being utilized routinely within the Department of Pathology at Carl Gustav Carus University Clinic Dresden. The system will therefore be used to grade all specimens. Since recent evidence indicates considerable observer variation that limits utility of the method (41), paraffin sections and/or digitized images will be sent from outside centers for review in Dresden. Additional pathologists at outside participating centers may also be invited to review paraffin sections and/or digitized images to check and ensure consistency of scores.

(xvi) **Patient follow-up:** To be carried out as part of Phase 4 in all patients with suspected or confirmed PPGLs and GEP tumors enrolled into the protocol. The nature of the follow-up differs according to whether or not a tumor has been confirmed according to the criteria outlined above and is decribed in more detail later in this protocol. Follow-up is essential for addressing all specific aims of this protocol.

(xvii) **MiRNA profiling:** For miRNA profiling, a 1 mL sample of blood is required, which will be taken from all subjects, including patients with PPGLs and GEP tumors during phases 1 and 4 of the study. After extraction of the RNA, miRNA detection will be performed in a 384-Well-Format using the the innovative Riboxx-Technology. The Riboxx-Technology used in this study for in

vitro profiling of miRNA is protected by 3 patent families (PCT WO 2007/012329, PCT EP2009/065131 and PCT EP2009/057119). The data from miRNA profiling provide the central component necessary for addressing specific aim #7.

(xviii) **Specimen banking:** Samples of plasma, serum, urine, and where available, tumor tissue and DNA, will be collected from all patients with monoamine-producing tumors and stored in -80^{o}C freezers. For purposes of control comparisons and establishing normal ranges of novel biomarkers, samples of serum, plasma and urine will also be collected for banking from all normotensive healthy volunteers and patients with primary hypertension. For additional comparison purposes, such samples will also be collected from a similar number (n=300) of randomly selected patients in whom the tumors are excluded. The total blood volume for banking of plasma, serum and DNA will not exceed 40 mL and will be collected according to the procedures outlined above. Urine specimens will be derived from 24-hour collections. Tumor specimens will be procured according to standard operating procedures, but only after initial examination by the on-duty pathologist. All samples for specimen banking are stored in coded tubes without any other attached information that would allow identification of the individual from whom the sample is collected. All specimens are stored in secured, monitored and alarmed freezers at -80^{o}C.

3.8.2 Flow plan of procedures for patients with suspected PPGLs

Additional tests and procedures beyond those outlined above may be carried out as clinically indicated, but in all such situations these tests and procedures are conducted ouside of this protocol. Such additional procedures include but are not limited to preoperative preparation of patients, all surgical procedures and any form of treatment (e.g., radiotherapy), all of which are not covered by this protocol. The procedures outlined above for patients with suspected and subsequently confirmed or excluded PPGLs will be carried out according to the visit plan and flow chart in figure 1, and further summarized below.

(i) **Initial Screening Tests:** Plasma and 24 hr urines are collected for measurements of plasma free metanephrines and urinary fractionated deconjugated and free metanephrines (Phase 1). These measurements will be used to compare diagnostic efficacy of the three tests as initial screening tests, either alone or in combination (specific aim #1).

(ii) **Patients with normal screening results for both plasma free and urinary deconjugated metanephrines:** If results for plasma free and urinary deconjugated metanephrines are normal, the tumor is considered excluded and no further testing for PPGLs is immediately required under the protocol. However, for entry of data into the final analysis, the presence of PPGL must be excluded independently by patient follow-up at a minimum of two years after initial screening (Phase 4). As outlined in the earlier section on “primary outcome parameters” this independent criterion for exclusion is essential for categorizing results of initial screening tests as true-negative or false-positive (specific aim #1). The majority of patients enrolled into the protocol (about 90%) are expected to follow this flow path.

(iii) **Patients with strongly positive results for initial screening tests of plasma free metanephrines:** Patients are designated as having biochemically confirmed PPGL if they are found to have strongly increased results for initial screening tests of plasma free metanephrines (i.e., more than 4-fold above the upper reference intervals) and an appropriate pattern of results for the corresponding urinary analytes. Patients in this group by-pass Phase 2 and are immediately entered into Phase 3 where they undergo complete biochemical profiling, imaging procedures, echocardiography and 24 hr BP monitoring. These procedures are carried out to address specific aims #3, #4 and #5. Only a small proportion of the patients enrolled into the protocol (less than 8%) are expected to follow this path.

(iv) **Patients with mild-to-moderately positive ("grey area") results for initial screening tests of plasma free metanephrines or with normal plasma free metanephrines and elevated urinary deconjugated metanephrines:** Patients with positive results for plasma free metanephrines, but below established confirmatory thresholds (i.e., less than 4-fold above the upper reference intervals) undergo confirmatory follow-up testing under Phase 2 of the protocol (specific aim #2). Confirmatory testing is also carried out in patients who show distinct miss-matches for initial screening tests of plasma and urinary metanephrines, including normal plasma free metanephrines, but elevated urinary deconjugated metanephrines Repeat tests of urine and plasma metanephrines are carried out to confirm initial elevated results under conditions that minimize all potential for false-positive results. If repeat tests are normal, a PPGL is considered excluded and the patient is treated as outlined above for the follow-up of normal test results. For patients with consistent elevations, follow-up testing includes measurements of plasma CgA, plasma catecholamines and 24-hr urinary catecholamines. For consistent elevations of plasma normetanephrine, follow-up testing also includes the clonidine suppression test, whereas for consistent elevations of urinary deconjugated normetanephrine or metanephrine, follow-up testing includes an overnight urinary collection. For negative results of follow-up testing, the tumor is considered excluded and the patient is treated as outlined above for the follow-up of normal test results. For positive results of confirmatory follow-up testing the presence of PPGL is designated as biochemically confirmed and patients proceed to Phase 3 where they undergo imaging procedures, echocardiography and 24 hr BP monitoring. About 12% of the patients enrolled into the protocol are expected to follow this flow path. Of these, only a small proportion of the total patients enrolled into the protocol are expected to proceed to Phase 3 (less than 5%). Most are expected to have the tumors biochemically excluded and proceed to Phase 4 (for follow-up 2-5 years later) as described above for patients with normal results for initial testing.

(v) **Tumor confirmation:** With the exception of certain cases of inoperable or malignant disease outlined below, PPGLs are confirmed by histopathological examination of resected tumor tissue. Histopathological confirmation is carried out by an experienced and qualified pathologist according to the procedures outlined above in section 3.8.1 xv. Occasionally patients are encountered with disease that is considered inoperable, this usually associated with malignancy (see also section 3.8.4 iv below). In such patients where the presence of catecholamine-

producing PPGLs are clear, biopsies are usually contra-indicated (they can lead to a hypertensive crisis). In such patients, in whom there is no tumor tissue available for pathological examination, confirmation of disease is established from a previous history of PPGL, confirmed by histopathology of the previously resected tumor(s), combined with functional imaging evidence of catecholamine-producing tumors (e.g., ^{123}I-MIBG scintigraphy) and independent biochemical evidence of catecholamine-overproduction. In those patients with inoperable disease in whom no tumor has previously been resected (e.g., those with malignant disease at first presentation) confirmation of disease may be based on the final two criteria outlined above; in such cases functional imaging evidence must be unequivocal and biochemical test results well outside of the physiological range (e.g., plasma normetanephrine > 400 pg/mL). Once PPGLs are confirmed, patients are invited to undergo genetic testing of tumor susceptibility genes.

(vi) **Genetic Counseling:** Before blood is collected for genetic testing, patients are provided the opportunity for genetic counseling. At Dresden this will be supervised by Dr. Barbara Klink in the Institute of Clinical Genetics (Medical Faculty Carl Gustav Carus, Technical University Dresden). German patients will be informed about the law concerning genetic diagnostics that was enacted in 2010. All patients with confirmed PPGLs will be evaluated for evidence of hereditary PPGLs from the taking of a detailed pedigree history of cancer, hypertension and cardiovascular disease. Patients will also undergo examinations for clinical stigmata that might point to a particular hereditary syndrome (e.g., dermal signs for NF1 or MEN 2; ocular signs for NF1 and VHL syndrome; thyroid and parathyroid examinations for MEN 2; evidence of CNS hemiangioblastomas, and tumors or cysts of the kidneys, pancreas and testis for VHL). Should a pathologic mutation be detected and confirmed (at Dresden, confirmation will be assisted utilizing the expertise available within the Institute of Clinical Genetics), then patients will be informed about the mutation and will receive futher detailed genetic counseling about the associated consequences of the particular mutation. Counseling will include an outline of the risks for recurrent PPGLs and other tumors and advice on appropriate periodic surveillance to detect disease at an early stage. Patients with identified *RET* mutations will be also be counseled concerning prophylactic throidectomy. Testing will be offered to other family members at risk for carrying the mutation. All such identified patients will be provided the opportunity for follow-up routine surveillance of PPGLs and other tumors that they may be at risk for developing.

3.8.3 Flow plan of procedures for patients with suspected GEP tumors

The aim of investigations involving patients with GEP tumors (specific aim #6) is to simply identify new biomarkers for these tumors that may be compared to existing biomarkers. Therefore the flow plan for patients enrolled into the study with suspected GEP tumors is much simpler than that for PPGLs. All standard diagnostic information at the time of initial testing and entry into the protocol is collected. Additional blood (40 mL maximum) and urine samples are collected for banking and storage at -80^{o}C for subsequent HPLC-coulometric detection and LC-MS/MS based monoamine metabolomic profiling.

Results of these analyses are compared with results of standard diagnostic tests (e.g., measurements of plasma CgA, urine 5HIAA and serotonin) according to whether the diagnosis of GEP tumors has been confirmed or excluded. Diagnosis is confirmed by histopathological analyses, either performed on surgically excised tumor specimens or from biopsy material. Diagnosis is excluded by patient follow-up as described below. The flow plan therefore simply involves two phases: 1. diagnostic screening and 2. disease verification and follow-up.

3.8.4 Patient follow-up

All patients with suspected or confirmed PPGLs or GEP tumors undergo follow-up. The timing and nature of the follow-up is dependent on whether tumors have been considered excluded by biochemical testing, whether tumors have been confirmed, and if so the presence of underlying mutations or malignant disease.

(i) **Patients with biochemically excluded PPGLs**: Follow-up of patients with biochemically excluded PPGLs is carried out between 2 to 5 years after initial enrollment in the study. Follow-up requires an initial phone conversation either directly with the patient or the patient's physician. If all signs, symptoms or medical problems that prompted initial suspicion of a PPGL have resolved, or if an alternative diagnosis that accounts for these signs, symptoms or medical problems has been established, then follow-up is complete and PPGL is considered excluded. The patient's involvement in the protocol is then considered complete. For persisting signs and symptoms, or unresolved relevant medical conditions for which there is no satisfactory alternative diagnosis, the patient is invited back to the clinic for another H&P and repeat biochemical testing by measurement of plasma free metanephrines. Negative biochemical test results on follow-up designate final exclusion of disease and the patient's involvement in the protocol is complete. Positive biochemical test results require further testing as described earlier. In the event that a final diagnosis confirming or excluding PPGL cannot be reached, or if the patient is lost to follow-up, then data from the patient are excluded from the final analyses and involvement of the patient in the protocol is considered complete.

(ii) **Patients with surgically resected PPGLs**: Patients with surgically resected tumors remain at risk for recurrent disease throughout life. Such disease may occur in up to 20% of patients after initial surgery in one or more of the following presentations: 1. local recurrence at the site of resection; 2. multifocal disease in the context of a hereditary syndrome; or 3. development of malignant disease defined as the presence of metastases at sites where chromaffin tissue is normally absent (e.g., bones, lungs, liver, lymph nodes). Thus, patients who have had their tumors removed by surgery should never be misinformed that their tumors are benign and that they have been cured; it is recommended that all patients should be counseled that a recurrence is possible and for that reason they should undergo periodic surveillance for such recurrence (4). Therefore, all patients with tumors identified under this protocol will receive annual follow-up over the course of the 5-year protocol that will include measurements of plasma metanephrines. All surgically treated patients with PPGLs will be followed-up as follows: Between 2 and 4 weeks after surgery patients will be seen in the outpatient department for follow-up visit #1. Blood

and/or urine will be sampled for measurements of plasma metanephrines to exclude residual or undiagnosed malignant disease. At 12 months, all patients with surgically resected PPGLs are scheduled for a second follow-up visit. In addition to tests of plasma metanephrines, patients will undergo follow-up echocardiography, EKG, 24 hour blood pressure monitoring and complete biochemical profiling (for comparison with results before resection of tumors to address specific aims #3, #4, and #5). Further follow-up at yearly intervals will be restricted to measurements of plasma free metanephrines. The patient's involvement in the protocol is considered complete at the end of the 5-year duration of the protocol. However, at that point all patient's are counseled to continue their yearly check-ups outside of the protocol.

(iii) **Patients with mutations of tumor susceptibility genes**: Patients with confirmed hereditary PPGLs or mutations of known tumor susceptibility genes undergo similar follow-up testing as described above. However, patients with SDHB or SDHD mutations also undergo MRI or CT scans of abdomen, thorax and neck (irrespective of symptoms or biochemical test results) to rule out non-functional paragangliomas. Annual surveillance for other tumors should also be carried out where necessary as mandated by the particular syndrome and recommended during genetic counseling (see section 3.8.2. vi). Such surveillance, however, is carried out outside of this protocol.

(iv) **Patients with inoperable PPGLs**: Among the expected 200 patients with confirmed PPGLs who will be enrolled into the protocol, approximately 15% (n=30) can be expected to have or develop inoperable malignant disease. Patients with inoperable disease are followed up at periodic intervals throughout the 5-years of the protocol to assess tumor burden and disease progression. Complete biochemical profiling to assess tumor burden and disease progression is carried out at 6 month intervals, whereas imaging studies to assess tumor burden and echocardiography and 24-hour blood pressure monitoring to assess progression of cardiovascular complications are carried out at yearly intervals. Although each patient's involvement is considered complete at the conclusion of the 5-year protocol, patients will be encouraged to continue periodic follow-up. Such continued assessments of disease progression can be particularly crucial for facilitating entry of patients into clinical trials of new therapies where baseline data of disease progression is often required before therapies can be commenced.

(v) **Patients with suspected GEP tumors**: Among patients suspected to have GEP tumors in who initial standard diagnostic testing fails to confirm disease, the diagnosis will be considered excluded only after patient follow-up 2 or more years after initial testing indicates that the patient is disease free. As for patients with suspected but biochemically excluded PPGLs, follow-up of patients suspected to have GEP tumors will be based at the outset on telephone interviews with the patient or patient's physician. If all signs, symptoms or medical problems that prompted initial suspicion of a GEP tumor have resolved, or if an alternative diagnosis that accounts for these signs, symptoms or medical problems has been established, then follow-up is complete and a GEP tumor is considered excluded. The patient's involvement in the protocol is then considered complete. For persisting signs and symptoms, or unresolved relevant medical

conditions for which there is no satisfactory alternative diagnosis, the patient is invited back to the clinic for repeat diagnostic testing. Negative test results on follow-up designate final exclusion of disease and the patient's involvement in the protocol is complete. Positive test results require further testing. In the event that a final diagnosis confirming or excluding as GEP tumor cannot be reached, or if the patient is lost to follow-up, then data from the patient are excluded from the final analyses and involvement of the patient in the protocol is again considered complete. Patients in who GEP tumors are confirmed received yearly follow-up over the 5–year course of the protocol. Follow-up under the protocol includes complete biochemical profiling and recording of all routine diagnostic tests.

3.9 STATISTICAL ANALYSES

The primary outcome parameters described above (# 3.2) will be used for stratification of patients into groups and subgroups, with statistical analyses directed at differences in secondary outcome parameters (# 3.3) among groups and subgroups.

(i) **Analysis of data**: Analysis of diagnostic test performance will employ standard measures of diagnostic sensitivity, specificity, and positive and negative predictive values at different pre-test prevalence's of disease. Reference intervals for analytes will be established from distributions of the measured variables in reference populations of normotensives and hypertensives using either non-parametric or parametric approaches as indicated by the nature of distributions. Analysis of receiver operating characteristic curves by logistic regression (for multiple analytes per test), with significance determined from areas under the curves (method of Hanley and McNeil) will provide the primary method to assess differences in diagnostic test performance. The above standard methods of analysis of diagnostic test performance depend on a binary approach for assessment of measured variables (i.e., whether a test result is positive or negative). This, however, ignores the additional important information available from the "continuous" nature of measured variables. Statistical methods will therefore be used to assess positive-predictive values and likelihood ratios of disease according to the magnitudes of increases in measured variables above upper reference intervals.

The different initial and follow-up testing strategies (e.g., urinary fractionated metanephrines followed by urinary catecholamines & CgA versus plasma free metanephrines followed by clonidine suppression testing etc) outlined in the flow diagram for patients with PPGLs (see Figure 1) will be assessed according to the results of diagnostic test performance to establish the most effective and efficient sequence and combination of tests for confirmation and exclusion of PPGLs.

Other group comparisons will be by t-tests, analyses of variance and chi-squared tests as appropriate. Analysis of variance in a general linear model will be used to test the relations between all factors and covariates and the specific patient groups. Logistic and linear regression analysis will be used to test the significance of characteristics to predict disease features or manifestations (e.g., symptoms, signs, metabolic and cardiovascular complications, specific

mutations, presence of malignancy). Cluster analysis will be used to identify and confirm the subgroups of patients according to disease presentation and test results. Discriminant analysis may be used to build a predictive model based on observed factors.

(ii) **Power analysis**: The planned total numbers of patients with and without PPGLs was calculated according to the method described by Eng (42) for diagnostic studies involving comparisons of two proportions according to the formula below:

$$\mathbf{N} = \frac{2 \times \left[Z_{crit} \times \sqrt{2p\,(1-p)} + Z_{pwr} \times \sqrt{P_1\,(1-P_1) + P_2(1-P_2)} \right]^2}{D^2}$$

In the above formula, P1 and P2 represent the two proportions to be compared, D = P1 – P2 (i.e., the minimum expected difference between the two proportions), p = P1+P2/2 (i.e., the average of the two proportions), and Z_{crit} and Z_{pwr} are cutoff points along the x axis of a standard normal probability distribution that demarcates probabilities matching the specified significance criterion and statistical power respectively. For the present analysis a significance criterion of 0.05 and a power of 0.90 were chosen. For comparisons involving three tests this requires Z_{crit} = 2.39 and Z_{pwr} = 0.842. The proportions P_1 and P_2 were based on hypothesized values for diagnostic accuracy. Diagnostic accuracy is defined as the total number of patients with true-positive and true-negative results divided by the total number of patient results. As such, estimates of diagnostic accuracy reflect both diagnostic sensitivity and specificity and take into account both groups of patients with and without disease. Due to the referral nature of patients tested for these tumors we assumed a relatively high pretest prevalence of 0.083333 (i.e., 1 patient with confirmed disease for ever 12 tested). We based our value of diagnostic accuracy for measurements of plasma free metanephrines on previously published values for diagnostic sensitivity of 98.6% and specificity of 89.3% (3). From these values and the assumed pretest prevalence of 0.83333, diagnostic accuracy (P_1) was calculated to be 0.901. We based our power analysis on the assumption that diagnostic measurements of urinary free metanephrines (the hypothesized next best test) would offer improved diagnostic specificity (84.0%) compared to previously published values for urinary fractionated deconjugated metanephrines (68.6%), and similar diagnostic sensitivity to plasma free metanephrines (98.6%). With these hypothesized indices of diagnostic sensitivity (98.6%) and specificity (84.0%), diagnostic accuracy of urinary free metanephrines (P_2) was hypothesized not be greater than 0.852. Using the above values in the power analysis it was calculated that N = 1914. This represents the expected number of patients required to establish a significant difference (at $P < 0.05$) in diagnostic accuracy for tests of plasma free metanephrines versus urinary free metanephrines. Using the same method outlined above this number of patients can also be calculated to be more than sufficient for establishing significant differences in diagnostic accuracy for tests of both plasma and urinary free metanephrines versus urinary fractionated deconjugated metanephrines.

The above statistical analyses was carried out by Prof Graeme Eisenhofer. An independent confirmation was performed by Uta Schwanebeck (Biometriker, Koordinierungszentrum für Klinische Studien Dresden, Medizinische Fakultät Carl Gustav Carus der Technischen Universität Dresden).

4. HUMAN SUBJECTS PROTECTIONS

.

4.1 BENEFITS

Patients with suspected PPGLs who are enrolled into the study undergo standardized procedures for their diagnostic work-up and follow-up that may convey some benefits beyond those available through regular care channels at other non-specialist centers. Patients in this study benefit from a detailed general and endocrinological evaluation, including the most up-to-date and unique biochemical studies. Patients with identified PPGLs are also offered genetic testing that might not always be available at non-specialist centers. They are also followed-up systematically for at least two years after initial evaluation. Patients are therefore more closely and thoroughly observed than otherwise may be the situation. The information gained from this study may affect current and future clinical management and treatment of individual patients with PPGLs and GEP tumors.

4.2 RISKS AND HAZARDS

There is no additional risk by the study set-up in itself. The risks and hazards are limited to the procedures that are already operative in clinical care:

Venipuncture for blood sampling: Blood sampling will be carried out under the usual precautions. Placement of intravenous lines may cause transient pain or bleeding under the skin complicated by bruising. Seldomly a patient may faint due to the stress of venipuncture. However, all patients will be supine during placement of intravenous lines and blood sampling. If the patient feels weak or dizzy during orthostasis, the treatment is to elevate the legs with subjects supine. The maximum total amount of blood drawn from adult patients will not exceed 40 ml per day (measurements of plasma catecholamines and metanephrines, genomic and proteomic profiling). For pediatric patients, no more than 3 ml/kg will be drawn in a single blood withdrawal and no more than 7 ml/kg will be drawn over any six-week period.

Clonidine suppression test: There is a risk of an exagerated blood pressure fall in some patients treated with clonidine. Patients typically become hypotensive starting one hour after the drug is taken. Severe hypotension may occur in some patients, particularly those taking beta-blockers or who have a history of postural dizziness or orthostatic intolerance. Large decreases in blood pressure are treated using i.v. fluids and legs elevated according to the Trendelenburg procedure. The clonidine suppression test will not be carried out in any patients taking tricyclic antidepressants or other drugs (e.g., mirtazapin) that would either compromise test results or that are contraindicated because of potential interactions. The clonidine test will not be carried out in pregnant women and children under the protocol; however, this does not exclude these subjects having the tests carried out as part of their normal medical care when this is clinically indicated.

Allergy to contrast media: There is a very small (< 0.5%) risk of development of a new allergy to contrast media. Exposure to contrast media in this protocol is part of clinically indicated procedures like CT, MRI and MIBG scanning.

Radiation exposure due to imaging procedures: Scanning that includes CT and nuclear medicine procedures involve exposure to radiation. Alternative imaging procedures that do not involve radioactivity (e.g., MRI) will be used preferably for any children and pregnant or lactating women enrolled in the protocol. Imaging studies involving radioactivity will not be carried out in pregnant women under the protocol. When clinically indicated, children with PPGLs may undergo ^{131}I-MIBG scintigraphy under the protocol, but they will not undergo CT or PET scanning under the protocol. Nevertheless, this does not exclude these subjects for eligibility for these imaging procedures as part of their normal medical care when this is clinically indicated. All scanning involving radiation will be carried out only as clinically indicated, and there is no additional radiation risk from participation in this protocol as compared to accepted clinical practice (see attached memo on radiation exposure under the protocol from Prof. Michael Laniado, Director of the Institute and Polyclinic for Diagnostic Radiology). In fact, it is likely that participation in the protocol will assist in minimizing exposure to radiation to many patients in whom tumors are suspected, but in whom initial biochemical evidence is insufficient for reliable diagnosis.

Collection of DNA and mutation testing: Analyses of DNA could have an impact on insurance coverage, employment decisions, and other aspects of the patient's life. These risks will be outlined to all subjects participating in the protocol as described in the consent documents and outlined during genetic counseling. To minimize these risks, samples of DNA will be stored pseudonymized in freezers contained in a secured building of participating Institutions. The samples will be inventoried and stored by codes defined by the principal investigator or his designates. The procedures itemized below will be followed to further minimize the chance that genetic information about a particular patient could impact on insurance coverage, employment decisions, or other aspects of the patient's life, against the patient's wishes or interests.

(i). Any information about specific genetic analyses to be carried out or results of subsequent genetic testing are to be conveyed directly to the patient by a member of the research team.

(ii) If the patient wishes to receive the results of the testing, then he or she sends a written, witnessed request for the results to one of the Investigators. After receipt of a written, witnessed request, results of the genetic analysis are sent directly to the patient.

(iii) If the patient requests and receives information about genetic analyses, the patient is informed by a qualified member of the research team about the meaning of the analyses and possible relevance to insurance coverage, employment decisions, or other aspects of the patient's life that would be against that particular patient's wishes (see section on Genetic Counseling 3.8.2. vi).

4.3 SUMMARY/CLASSIFICATION OF RISK:BENEFIT

The subjects in this study are subject to varying risk according to the different procedures that they are subject to. However, this is an observational cohort study that does not involve experimental

therapeutic interventions or drugs. All procedures and tests carried out on patients under this protocol are commonly used in routine clinical care. Thus, any risk associated with participation in this protocol is not expected to exceed that associated with routine clinical care.

There is some risk from experimental procedures in non-patient subjects recruited into the protocol as primary hypertensives or healthy normotensive volunteers for purposes of establishing diagnostic reference intervals or for comparison with patient populations. However, this risk is associated with blood sampling and is therefore minimal; there also may be some benefit to such volunteers associated with entry criteria examinations (e.g., history and physicals) prior to acceptance into the protocol.

All subjects in this study benefit from a careful, expert medical history and physical examination and screening clinical laboratory tests. In many patients these evaluations will provide a diagnosis that might lead to an effective treatment or cure outside of the protocol. Thus, in these patients there may be some direct benefit to participating in the study. In all others there is likely to be no direct benefit, but participation may yield generalizable knowledge that could ultimately lead to the improved diagnostic tests for PPGLs and GEP tumors and development of more effective treatments for malignant PPGLs. Such knowledge could impact the future health and well being of the same or other patients suffering from the conditions studied under this protocol.

4.4 DATA SAFETY AND MONITORING

Data and safety will be monitored by the study leaders at each participating site. All raw data, documentation, protocols, final reports, and data analyses will be retained indefinitely, but for no less than two years following completion of the study. Subjects participating in studies will not be identified by name in any publically-available written or oral reports of study findings. Confidentiality of patient information will be maintained at all times. Paper records and case report forms will be maintained in locked cabinets, rooms, or in computer files protected through the use of computer passwords. Only authorized personnel will have access to this data. Human specimens are stored under codes in locked freezers and laboratories. An inventory system is in place that links coded samples to identified patients and patient data under the control of the PI, listed co-investigators and those under PI's direct supervision charged with maintaining inventories and specimen banks. Only those persons can make the linkage between coded specimens and human data. Inventories will include records for tracking specimens used in collaborative research. Patient data are stored in locked cabinets, offices, and password protected computers and databases.

Recording, storage, disclosure, and analysis of personal data of the participants within this clinical study are in accordance with legal requirements (Sächsisches Datenschutzgesetz, Bundesdatenschutzgesetz). The participant has to agree on the handling of his/her data within the informed consent form. The participant has to be informed about:

- data are recorded electronically, will be handled confidentially, and disclosed to others (e.g., off-site investigators, local and federal authorities, independent ethical committee, European data bank) only pseudonymized.
- persons who are authorized to inspect the clinical study can have insight into participant related data. These persons have to handle the data confidentially. The clinical investigator is dispensed from his/her medical confidentiality towards these persons.
- the written consent for data recording and documentation during this clinical study is irreversible. When a participant withdraws the written consent, all data that are documented so far can be used pseudonymized to analyse the study.

4.5 Adverse Event Documentation and Reporting

Individual subjects will be monitored for expected and unexpected effects of procedures and adverse events by study investigators. Serious adverse events are defined as any untoward medical occurrence that (1) results in death, (2) is life threatening, (3) requires (or prolongs) hospitalization, (4) causes persistent or significant disability/incapacity, (5) results in congenital anomalies or birth defects, or (6) are in the judgment of the investigators represents a significant hazard. The Study Leaders at each participating site will report serious adverse events verbally and in writing as soon as possible to the Chair of the local Ethics Committee within 7 calendar days for death or life-threatening adverse events, and within 15 days for all other serious adverse events. For serious adverse events, the patient is treated for immediate medical problems (e.g., anaphylactic reaction) in an appropriate setting (e.g., ICU). Adverse events are defined as any unfavorable and unintended diagnosis, symptom, sign (including an abnormal laboratory finding), syndrome, or disease, which either occurs during the study, having been absent at baseline, or if present at baseline, appears to worsen. All adverse events are documented in the participant's chart and in the Case Report Form. Adverse events will be reported after intake of the first dose of study medication. The participant will be followed until remission of the symptoms. All adverse events (serious and non-serious, expected and unexpected) will be reported in written form to the Ethics Committee at the time of the next scehuled review of the protocol. The following information is included in an adverse event report: a concise description of the event, the onset date of the event, the duration of the event, the severity of the event, and the type of episode, the relationship of the event to the study drug and the outcome of the event. In the unlikely event of untoward or unexpected outcomes occur, the patient will be treated for immediate medical problems (e.g., anaphylactic reaction) in an appropriate setting (e.g., ICU).

5. ETHICAL, LEGAL AND ADMINISTRATIVE ASPECTS

5.1 Multicenter Involvement

As a multicenter study, this protocol has been adapted by the other two participating centers for submission to the Independent Ethics Committees (IECs) of those centers. Other centers may also be invited to participate utilizing adaptations of this protocol. Recruitment of subjects, sample and data

collections and investigations are carried out in a standardized fashion according to this protocol and existing standard operating procedures at all participating centers.

Blood and urine specimens for measurements of analytes in the screening and follow-up diagnostic tests used for purposes of addressing the specific aims of the study will as a general rule be analyzed at a single Central Reference Laboratory (CRL) at the Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Dresden. Some of these assays, however, may also be carried out within the individual laboratories at participating centers, but only in situations in which those laboratories participate in a common inter-laboratory Quality Assurance Program and where comparisons of assays performed as part of this program indicate good agreement (not greater than a 10% difference in accuracy – bias).

Importantly, however, the assays performed at the CRL will not serve to replace already in place standard of care diagnostic tests carried out at off-site centers. Similarly, the information from the assays performed at the CRL will not serve to supplant clinical decision-making processes at off-site centers carried out according to the information derived from independent diagnostic tests carried out at those centers. The results of diagnostic tests carried out within the CRL will nevertheless be provided back to investigators at other participating centers, and this information will be used for purposes of follow-up testing according to the protocol.

Metabolomic profiling by LC-MS/MS will also be carried out at the Institute of Clinical Chemistry and Laboratory Medicine (University Hospital Carl Gustav Carus, Dresden) from coded specimens collected, stored and shipped in batches from other participating centers. Similarly, MiRNA profiling of serum samples will also be carried out at a single center (by Dr. Jacques Rohayem, at Riboxx GmbH) using coded specimens. Banking of samples (e.g., tumor tissue, plasma, urine, DNA) will be carried out at each participating center according to standardized procedures. Any subsequent exchange of samples or data will be carried out in a pseudonymized fashion using coded specimen containers or data forms.

5.2 OFF-SITE ENROLLMENT

Currently blood and urine specimens for laboratory accredited diagnostic testing of suspected monoamine-producing tumors are being received not only from within the University Medical Center, but also from other centers throughout Germany. The study will take advantage of this already existing network by offering invitations for participation in the protocol to the clinicians and patients at these outside centers. As outlined in the section above on “Multicenter Involvement”, some centers with both clinical interest and sufficiently large volumes of patients are invited to utlize adaptations of this protocol for submission to their own IEC. For other centers, this protocol allows offsite enrollment of patients for purposes of initial screening for PPGLs or GEP tumors. In this situation consent forms, Pheochromocytoma Research Support Organization (PRESSOR) information forms and written instructions for sampling and shipping of specimens according to established SOPs will be made available to clinicians at outside centers. Those clinicians will obtain written informed consent and ensure that all Phase 1 procedures are carried out according to the protocol. Samples will be sent to either the Central Reference Laboratory in Dresden. Results of the off-site testing for accredited diagnostic tests (i.e., plasma metanephrines, urinary fractionated metanephrines) will be returned to

the clinician. Where results are negative no further action is required other than patient follow-up at the appropriate interval after initial testing. In situations of slightly increased positive test results, further instructions will be made available for follow-up testing (i.e., clonidine suppression tests, urinary tests, plasma tests) according to Phase 2 procedures. Where initial screening or follow-up testing strongly indicates the presence of a PPGL the patient will be invited to one of the participating centers for the Phase 3 procedures required for disease characterization.

5.3 RESPOSIBILITIES OF THE PRINCIPAL INVESTIGATOR

The sponsor (Medical Faculty) and the principal clinical investigator assure that the clinical study is performed in accordance with:

- ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996,
- Declaration of Helsinki concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects, Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996, Edinburgh, 2000),
- German law (AMG and GCP guidelines 2004).

This study must be carried out in compliance with the protocol and in accordance with all associated standard operating procedures. The **investigator** agrees, when signing the protocol, to adhere to the instructions and procedures described in it and thereby to adhere to the principles of Good Clinical Practice that it conforms to.

The responsibilities of the investigator include:

- execution of the study plan,
- sufficient time and capacity to perform the clinical study,
- correct collection and documentation of study related data and reporting,
- provision of data for monitoring and for audits/inspections,
- ensuring strict confidentiality and requesting similar confidentiality from her/his staff concerning information about participants and information provided by the sponsor. Study documents will be stored appropriately to ensure their confidentiality.
- providing financial disclosure.

The clinical investigator has full responsibility for the conduct of the clinical study in the designated study center.

5.4 APPROVAL AND START OF THE STUDY PROTOCOL

Before implementing this study, the protocol, the proposed informed consent forms, and other information to patients must be reviewed by the Independent Ethics Committee (IEC) of the Medical Faculty of the Technical University of Dresden. A signed and dated statement that the protocol and informed consent have been approved by the IEC must be provided to the Study Leader before the protocol can be initiated. Any amendments to the protocol, other than administrative ones, must be approved by this committee. Once approved, the study will be initiated.

5.5 CONSENT DOCUMENTS AND PROCESS

The study leaders or those listed as co-investigators will obtain informed consent using forms appended to this protocol. Off-site consenting requires in addition to the patients signature, both the signature of the clinician at the off-site center and the study leader or a coinvestigator at one of the three participating centers listed in this protocol. Before inclusion in the clinical study, each patient must receive an explanation about the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved, and any discomfort it may entail. Each subject will also receive a written explanation of the purposes, procedures, and potential hazards of the project. Each patient must be informed that participation in the study is voluntary and that she/he may withdraw from the study at any time and that withdrawal of consent will not affect her/his subsequent medical treatment or relationship with the treating physician. This informed consent should be given by means of a standard written statement, written in non-technical language common to the country of the participating center. The patient should read and consider the statement before signing and dating it. If written consent is not possible, oral consent can be obtained if witnessed by a signed statement from one or more persons not involved in the study, mentioning why the patient was unable to sign the form. For children, signed parent consent must be obtained. No patient can enter the study before his/her informed consent has been obtained. For patients who do not speak or understand the local language an interpretor will be enlisted to facilitate the consent process and assist with any other communication requirements. The informed consent form is signed and dated by the participant and by the investigator (and clinician at off-site centers). One original copy of the consent form is filed by the study leaders at the particular center where the participant was enrolled. Another original copy is provided to the participant.

This protocol includes procedures that allow consenting of patients in an off-site setting, primarily to obtain blood samples for screening purposes. After initial contact with the patient or referring physician, a package is sent with the information and forms required for participation in the study, including instructions for drawing and shipping samples and the protocol consent form. The patient is provided the opportunity of discussing the protocol by telephone with a member of the research team. After any questions have been answered to the patient's satisfaction, the patient signs the consent form, as documented by the signature of the patient's clinician. The original signed and dated original consent form is sent to the principal investigator at one of the participating main study centers and signed and dated on receipt by a member of the research team. A notation about the off-site nature of the screening is provided in the margin of the signature page to explain any differences in the dates that patients and members of the research team provided their signatures.

5.6 FINANCIAL COMPENSATION

Healthy normal volunteers will be offered €50.00 for their participation and compensation for time involved in providing a blood sample and urine samples for use in establishing or updating reference intervals. Patients enrolled into the hypertensive reference group and all patients enrolled because of

suspected or established PPGLs or GEP tumors will not receive financial compensation under the protocol.

5.7 Study Discontinuation (Drop-Outs and Withdrawals)

Participation in the study is voluntary. A subject has the right to withdraw from the study at any time for any reason. If she or he chooses to withdraw, the investigator will be informed immediately.

The principle or medically responsible investigator at each participating site has the right to terminate the participation of any subject at any time, if she or he deems it in the participant's best interest. The reason and circumstances for study discontinuation will be documented in the participant's case Report Form.

Reasons for study discontinuation might be:,

- occurrence of a concomitant disease,
- withdrawal of consent for personal reasons
- relevant non-compliance with the protocol.

5.8 Amendments

Any change or addition to this protocol requires a written protocol amendment that must be approved and signed by the investigator before implementation. Amendments significantly affecting the safety of patients, the scope of the investigation, or the scientific quality of the study require approval by the Independent Ethics Committee (IEC) of the Medical Faculty of the Technical University of Dresden. A copy of the written approval of the IEC must then become part of the amended protocol.

These requirements for approval should in no way prevent any immediate action from being taken by the investigator in the interests of preserving the safety of all patients included in the study. If an immediate change to the protocol is felt to be necessary by the investigator and is implemented by him/her for safety reasons, the IEC at the center should be informed within 10 working days. Amendments affecting only administrative aspects of the study (e.g., changes in staff designated with patient interactions) do not require formal protocol amendments or IEC approval, but the IEC must be kept informed of such administrative changes.

5.9 Execution of the Study

Enrollment of German patients into the study will be centered in the Medical Clinic III of the Carel Gustav Carus University Clinic Dresden, but may also occur at other centers in Germany where this protocol has been adapted, submitted to and approved by the IECs at those centers. For purposes of initial biochemical screening, patients may be enrolled at off-site centers, particularly those where there is already a program in place. Other study procedures outlined above may also be carried out at off-site centers, or alternatively where appropriate patients may be referred to Dresden.

5.10 Time Span of the Protocol

1-8-2010 – 1-8-2013 Completion of enrollment of all subjects and Phases 1 through 3

1-8-2013 – 1-8-2015 Completion of follow-up and Phase 4.

An der Studie beteiligtes ärztliches und nicht-ärztliches Personal:

	Studienfunktion	**Beruflicher Hintergrund**
Dr. Roland Därr	Clinical Scientist	Assistenzarzt/Endokrinologie
Prof. Dr. Lorenz C. Hofbauer	Leiter endokrinologische Ambulanz	Bereichsleiter Endokrinologie
Dr. Mathias Gruber	Facharzt Innere Medizin	Assistenzarzt/Endokrinologie
Dr. Elena Tsourdi	Assistenzärztin Innere Medizin	Assistenzarzt/Endokrinologie
Frau Sonja Beutel	Ambulanzschwester	Endokrinologie
Frau Beate Kindel	MTA (Endokrinologische Funktionsdiagnostik)	Endokrinologie
Frau Susann Maier	Studienschwester	Endokrinologie
Dr. Klaus Zöphel	Facharzt Nuclear Medicine	Nuclear Medicine

6 SIGNATURES AND AGREEMENT WITH THE PROTOCOL

We, the undersigned, agree to conduct this study according to the above protocol. In addition, we agree that the trial will be carried out in accordance with Good Clinical Practice (GCP), with the Declaration of Helsinki and with the laws and regulations of the country in which the study takes place.

Principal Investigator: ....
Prof. Dr. Stefan Bornstein Date

Study coordination: ....
Prof. Graeme Eisenhofer PhD Date

Associate Clinical Investigators (Carl Gustav Carus University Clinic Dresden, Dresden)

We, the undersigned, agree to conduct this study according to this protocol. We commit ourselves to treat, to follow-up, and to document all included participants according to the study protocol.

Prof. Dr. Lorenz Hofbauer ..... Date .....

Prof. Dr. Hans-Detlev Saeger ..... Date .....

Prof. Dr. Jörg Kotzerke ..... Date .....

Prof. Dr. Gustavo Baretton ..... Date .....

Prof. Dr. Gabriele Siegert ..... Date .....

Prof. Dr. Angela Hübner ..... Date .....

Dr. Roland Därr ..... Date .....

PD Dr. Jacques Rohayem ..... Date .....

Prof. Dr. Michael Laniado ..... Date .....

Dr. Barbara Klink ..... Date .....

7. REFERENCES

1. **Lenders JW, Eisenhofer G, Mannelli M, Pacak K** 2005 Phaeochromocytoma. Lancet 366:665-675
2. **Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A** 2008 Gastroenteropancreatic neuroendocrine tumours. Lancet Oncol 9:61-72
3. **Lenders JW, Pacak K, Walther MM, Linehan WM, Mannelli M, Friberg P, Keiser HR, Goldstein DS, Eisenhofer G** 2002 Biochemical diagnosis of pheochromocytoma: which test is best? Jama 287:1427-1434
4. **Pacak K, Eisenhofer G, Ahlman H, Bornstein SR, Gimenez-Roqueplo AP, Grossman AB, Kimura N, Mannelli M, McNicol AM, Tischler AS** 2007 Pheochromocytoma: recommendations for clinical practice from the First International Symposium. Nat Clin Pract Endocrinol Metab 3:92-102
5. **Eisenhofer G, Keiser H, Friberg P, Mezey E, Huynh TT, Hiremagalur B, Ellingson T, Duddempudi S, Eijsbouts A, Lenders JW** 1998 Plasma metanephrines are markers of pheochromocytoma produced by catechol-O-methyltransferase within tumors. J Clin Endocrinol Metab 83:2175-2185
6. **Eisenhofer G, Huynh TT, Hiroi M, Pacak K** 2001 Understanding catecholamine metabolism as a guide to the biochemical diagnosis of pheochromocytoma. Rev Endocr Metab Disord 2:297-311
7. **Eisenhofer G, Lenders JW, Goldstein DS, Mannelli M, Csako G, Walther MM, Brouwers FM, Pacak K** 2005 Pheochromocytoma catecholamine phenotypes and prediction of tumor size and location by use of plasma free metanephrines. Clin Chem 51:735-744
8. **Eisenhofer G, Goldstein DS, Sullivan P, Csako G, Brouwers FM, Lai EW, Adams KT, Pacak K** 2005 Biochemical and clinical manifestations of dopamine-producing paragangliomas: utility of plasma methoxytyramine. J Clin Endocrinol Metab 26:1925-1939
9. **Neumann HP, Pawlu C, Peczkowska M, Bausch B, McWhinney SR, Muresan M, Buchta M, Franke G, Klisch J, Bley TA, Hoegerle S, Boedeker CC, Opocher G, Schipper J, Januszewicz A, Eng C** 2004 Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. Jama 292:943-951
10. **Amar L, Bertherat J, Baudin E, Ajzenberg C, Bressac-de Paillerets B, Chabre O, Chamontin B, Delemer B, Giraud S, Murat A, Niccoli-Sire P, Richard S, Rohmer V, Sadoul JL, Strompf L, Schlumberger M, Bertagna X, Plouin PF, Jeunemaitre X, Gimenez-Roqueplo AP** 2005 Genetic testing in pheochromocytoma or functional paraganglioma. J Clin Oncol 23:8812-8818
11. **Eisenhofer G, Huynh TT, Elkahloun A, Morris JC, Bratslavsky G, Linehan WM, Zhuang Z, Balgley BM, Lee CS, Mannelli M, Lenders JW, Bornstein SR, Pacak K** 2008 Differential expression of the regulated catecholamine secretory pathway in different hereditary forms of pheochromocytoma. Am J Physiol Endocrinol Metab 295:E1223-1233
12. **Eisenhofer G, Walther MM, Huynh TT, Li ST, Bornstein SR, Vortmeyer A, Mannelli M, Goldstein DS, Linehan WM, Lenders JW, Pacak K** 2001 Pheochromocytomas in von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2 display distinct biochemical and clinical phenotypes. J Clin Endocrinol Metab 86:1999-2008
13. **Eisenhofer G, Lenders JW, Timmers H, Mannelli M, Siegert G, Bornstein SR, Pacak K** 2008 Biochemical profiling of chromaffin cell tumors: guide for genotyping and prediction of malignancy. J Hypertens 22:S3
14. **Timmers HJ, Kozupa A, Eisenhofer G, Raygada M, Adams KT, Solis D, Lenders JW, Pacak K** 2007 Clinical presentations, biochemical phenotypes, and genotype-phenotype correlations in patients with succinate dehydrogenase subunit B-associated pheochromocytomas and paragangliomas. J Clin Endocrinol Metab 92:779-786
15. **Eisenhofer G, Bornstein SR, Brouwers FM, Cheung NK, Dahia PL, De Krijger RR, Giordano TJ, Greene LA, Goldstein DS, Lehnert H, Manger WM, Maris JM, Neumann HP, Pacak K, Shulkin BL, Smith DI, Tischler AS, Young Jr WF** 2004 Malignant pheochromocytoma: current status and initiatives for future progress. Endocr Relat Cancer 11:423-436
16. **Brouwers FM, Eisenhofer G, Tao JJ, Kant JA, Adams KT, Linehan WM, Pacak K** 2006 High frequency of SDHB germline mutations in patients with malignant catecholamine-producing paragangliomas: implications for genetic testing. J Clin Endocrinol Metab 91:4505-4509

17. **Pollard PJ, Briere JJ, Alam NA, Barwell J, Barclay E, Wortham NC, Hunt T, Mitchell M, Olpin S, Moat SJ, Hargreaves IP, Heales SJ, Chung YL, Griffiths JR, Dalgleish A, McGrath JA, Gleeson MJ, Hodgson SV, Poulsom R, Rustin P, Tomlinson IP** 2005 Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. Hum Mol Genet 14:2231-2239
18. **Denko NC** 2008 Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer
19. **Kim JW, Dang CV** 2006 Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 66:8927-8930
20. **Hsu PP, Sabatini DM** 2008 Cancer cell metabolism: Warburg and beyond. Cell 134:703-707
21. **Pacak K, Eisenhofer G, Goldstein DS** 2004 Functional imaging of endocrine tumors: role of positron emission tomography. Endocr Rev 25:568-580
22. **Gatenby RA, Gillies RJ** 2004 Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4:891-899
23. **Kroemer G, Pouyssegur J** 2008 Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13:472-482
24. **Feldman JM** 1985 Increased dopamine production in patients with carcinoid tumors. Metabolism 34:255-260
25. **Sharma BK, Smits BJ, Robinson R, Burns S, Trounson EN** 1984 Carcinoid tumour secreting dopa. Br Med J (Clin Res Ed) 288:1111-1112
26. **Nehar D, Lombard-Bohas C, Olivieri S, Claustrat B, Chayvialle JA, Penes MC, Sassolas G, Borson-Chazot F** 2004 Interest of Chromogranin A for diagnosis and follow-up of endocrine tumours. Clin Endocrinol (Oxf) 60:644-652
27. **Modlin IM, Latich I, Zikusoka M, Kidd M, Eick G, Chan AK** 2006 Gastrointestinal carcinoids: the evolution of diagnostic strategies. J Clin Gastroenterol 40:572-582
28. **de Herder WW** 2007 Biochemistry of neuroendocrine tumours. Best Pract Res Clin Endocrinol Metab 21:33-41
29. **Eisenhofer G, Goldstein DS, Walther MM, Friberg P, Lenders JW, Keiser HR, Pacak K** 2003 Biochemical diagnosis of pheochromocytoma: How to distinguish true- from false-positive test results. J Clin Endocrinol Metab 88:2656-2666
30. **Algeciras-Schimnich A, Preissner CM, Young WF, Jr., Singh RJ, Grebe SK** 2008 Plasma chromogranin A or urine fractionated metanephrines follow-up testing improves the diagnostic accuracy of plasma fractionated metanephrines for pheochromocytoma. J Clin Endocrinol Metab 93:91-95
31. **Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C** 2002 Germ-line mutations in nonsyndromic pheochromocytoma. N Engl J Med 346:1459-1466
32. **Bornstein SR, Gimenez-Roqueplo AP** 2006 Genetic testing in pheochromocytoma: Increasing importance for clinical decision-making. Ann NY Acad Sci 1073:94-103
33. **Galluzzi L, Larochette N, Zamzami N, Kroemer G** 2006 Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene 25:4812-4830
34. **Pan JG, Mak TW** 2007 Metabolic targeting as an anticancer strategy: dawn of a new era? Sci STKE 2007:pe14
35. **Eisenhofer G, Rivers G, Rosas AL, Quezado Z, Manger WM, Pacak K** 2007 Adverse drug reactions in patients with phaeochromocytoma: incidence, prevention and management. Drug Saf 30:1031-1062
36. **Peaston RT, Lennard TW, Lai LC** 1996 Overnight excretion of urinary catecholamines and metabolites in the detection of pheochromocytoma. J Clin Endocrinol Metab 81:1378-1384
37. **Timmers HJ, Kozupa A, Chen CC, Carrasquillo JA, Ling A, Eisenhofer G, Adams KT, Solis D, Lenders JW, Pacak K** 2007 Superiority of fluorodeoxyglucose positron emission tomography to other functional imaging techniques in the evaluation of metastatic SDHB-associated pheochromocytoma and paraganglioma. J Clin Oncol 25:2262-2269
38. **Tischler AS** 2008 Pheochromocytoma and extra-adrenal paraganglioma: updates. Arch Pathol Lab Med 132:1272-1284
39. **van Nederveen FH, Gaal J, Favier J, Korpershoek E, Oldenburg RA, de Bruyn EM, Sleddens HF, Derkx P, Riviere J, Dannenberg H, Petri BJ, Komminoth P, Pacak K, Hop WC, Pollard PJ, Mannelli M, Bayley JP, Perren A, Niemann S, Verhofstad AA, de Bruine AP, Maher ER, Tissier F, Meatchi T, Badoual C, Bertherat J, Amar L, Alataki D, Van Marck E, Ferrau F, Francois J, de Herder WW, Peeters MP, van Linge A, Lenders JW,**

Gimenez-Roqueplo AP, de Krijger RR, Dinjens WN 2009 An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. Lancet Oncol 10:764-771

40. **Thompson LD** 2002 Pheochromocytoma of the Adrenal gland Scaled Score (PASS) to separate benign from malignant neoplasms: a clinicopathologic and immunophenotypic study of 100 cases. Am J Surg Pathol 26:551-566
41. **Wu D, Tischler AS, Lloyd RV, DeLellis RA, de Krijger R, van Nederveen F, Nose V** 2009 Observer variation in the application of the Pheochromocytoma of the Adrenal Gland Scaled Score. Am J Surg Pathol 33:599-608
42. **Eng J** 2003 Sample size estimation: how many individuals should be studied? Radiology 227:309-313