Faculty of Medicine and Dentistry

Palacký University Olomouc

Doctoral study programme

Internal Medicine

Pheochromocytoma in Mice and Men

Jan Schovánek, MD

Supervisor: Associated professor Zdeněk Fryšák, MD, CSc.

Department of Internal Medicine III – Nephrology, Rheumatology and Endocrinology, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic

Expert tutor: Professor Karel Pacak, MD, PhD, DSc, FACE

Section on Medical Neuroendocrinology, Developmental Endocrine Oncology and Genetics Affinity Group, Eunice Kennedy Shriver National Institute of Child Health & Human Development, National Institutes of Health, Bethesda, Maryland, United States of America

Acknowledgement:

Firstly, I would like to express my sincere gratitude to my supervisor associated professor Zdeněk Fryšák, MD, CSc. for the continuous support of my Ph.D. study and related research, for his patience, motivation, and immense knowledge.

My sincere thanks also goes to my expert tutor professor Karel Pacak, MD, PhD, DSc, FACE who gave me the opportunity to join his team at National Institutes of Health, Bethesda, USA for his insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives, who gave access to the laboratory and research facilities. Without his precious support it would not be possible to conduct this research.

To professor Josef Zadražil, MD, CSc. and professor Vlastimil Ščudla, MD, CSc.

I thank my fellow labmates for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last years.
Thank you Paula, Petra and Raghu.

Last but not the least, I would like to thank my family: my parents Hana Schovánková a Petr Schovánek, sister Kateřina for supporting me spiritually throughout writing this thesis and my life.

Declaration of Authorship:

I hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. No other person's work has been used without due acknowledgement in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

Table of Contents

I. Theoretical Part

1. **Commentary**
2. **Pheochromocytoma/paraganglioma**
3. **Epidemiology**
4. **Genetics**
5. **Clinical Manifestations**
6. **Differential Diagnosis**
7. **Biochemical Diagnosis**
8. **Localization of pheochromocytoma**
9. **Treatment**
10. **Medical Therapy and Preparation for Surgery**
11. **Operative and Postoperative Management**
12. **Hypertensive Crisis**
13. **Malignant Pheochromocytoma**
14. **Prognosis and Monitoring**
15. **Perspectives**
16. **References**
17. **Plots and Figures**

II. Clinical Part

1. **Commentary**
2. **Introduction**
3. **Patients**
4. **Results**
5. **Discussion**

6. **References**
7. **Plots and Figures**

- **Pdf version of the published article**

III. Experimental Part

1. **Commentary**
2. **Introduction**
3. **Material and Methods**
4. **Results**
5. **Discussion**
6. **References**
7. **Figures and Tables**

- **Pdf version of the published article**

IV. List of shortcuts

V. Published articles with IP, with direct connection to the topic of dissertation; as a co-author

1. **Combination of 13-cis retinoic acid and Lovastatin: marked anti-tumor potential in vivo in a pheochormocytoma allograft model in female athymic nude mice**

    - Nölting S, Giubellino A, Tayem Y, Young K, Lauseker M, Bullova P, **Schovanek J**, Anver M, Fliedner S, Korbonits M, Göke B, Vlotides G, Grossman A, Pacak K.
    - Published in *Endocrinology*, IF 2014: 4.503

2. **High-Throughput Screening for the Identification of New Therapeutic Options for Metastatic Pheochromocytoma and Paraganglioma**

    - Giubellino A, Shankavaram U, Bullova P, **Schovanek J**, Zhang Y, Shen M, Patel N, Elkahloun A, Lee MJ, Trepel J, Ferrer M, Pacak K.
    - Published in *PLOS one*, IF 2014: 3.234

VI. Published articles with IP, without direct connection to the topic of dissertation; as a co-author

1. **Insulin-like Growth Factors in a clinical setting: Review of IGF-I**

    - Frysak Z, **Schovanek J**, Iacobone M, Karasek D
    - Published in *Biomedical papers of the Medical Faculty of the University Palacky*, IF 2015: 0.924

2. **Case report of ovarian goiter as a rare cause of hyperthyroidism**

    - Frysak Z, **Schovanek J**, Halenka M, Metelkova I, Duskova M, Karasek
    - Accepted for publication in Acta Endocrinologica (Buc), 03/8/2016, IF 2015: 0.268
    - Pfd version is not available, yet

3. **Ultrasound-guided Percutaneous Ethanol Injection Therapy in a 92 year-old patient with parathyroid adenoma and with a history of total thyroidectomy for papillary thyroid carcinoma**

    - Halenka M, Frysak Z, Koranda P, Schovanek J
    - Accepted for publication in Acta Endocrinologica (Buc), 27/7/2016, IF 2015: 0.268
    - Pfd version is not available, yet

I. Theoretical Part

PHEOCHROMOCYTOMA

Method of Jan Schovanek, MD; and Karel Pacak, MD, PhD, DrSc

Co-author: Tobias Engel, MD

Published:

1) Bope ET, Kellerman RD. Conn's Current Therapy 2013: Expert Consult: Online. Elsevier Health Sciences, 2012, page 806 - 815
2) Bope ET, Kellerman RD. Conn's Current Therapy 2015: Expert Consult: Online. Elsevier Health Sciences, 2014, page 800 - 810

1. Commentary:

This introduction part of my work is based upon a chapter in the textbook „Conn's Current Therapy" published in paper in 2013 and 2015 with online text. Prof Pacak offered me to take over the authorship after Tobias Engel who was his previous co-author of this educative text. In both editions my goal was to bring the text up to date with the most recent publications. For a long time the newest recommendations regarding diagnosis, treatment and patients follow-up were presented as expert statements as a results of expert meeting first held in Bethesda, MD, USA in October 2005 as "First International Symposium on Pheochromocytoma (ISP). This pioneering meeting was followed by 3 consecutive meetings held in United Kingdom, France and Japan.

Apart from state of art recommendation following these international symposia the Endocrine Society established a task force developing evidence-based guideline using the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) system to describe both the strength of recommendations and the quality of evidence. Part Perspectives was added as a last part of this doctoral thesis and should review the latest development and future perspectives in the field of pheochromocytoma and paraganglioma.

2. Pheochromocytoma/paraganglioma

The current (2004) WHO classification of endocrine tumors defines pheochromocytoma (for the purpose of this text, the term pheochromocytoma also refers to paraganglioma unless otherwise specified), as a tumor arising from catecholamine-producing chromaffin cells in the adrenal medulla. Closely related paragangliomas are divided into two groups: those arising from parasympathetic-associated tissues and those that arise from sympathetic associated chromaffin tissue. Sympathetic paragangliomas were formerly designated as extra-adrenal pheochromocytomas. Pheochromocytomas and paragangliomas are characterized by the synthesis, metabolism, storage, and usually, but not always, secretion of catecholamines.

Parasympathetic paragangliomas are mainly located along the cranial and vagus nerves. Glomus or carotid body tumors, for example head and neck paragangliomas can be locally invasive but rarely develop metastases and are usually nonsecretory.

Sympathetic paragangliomas mainly arise in the abdomen from chromaffin tissue neighboring sympathetic ganglia. Less often, they originate from the pelvis and infrequently from the mediastinum (2%) and neck (1%). In the abdomen, they often derive from the organ of Zuckerkandl, a collection of chromaffin tissue around the origin of the inferior mesenteric artery (Figure 1).

3. Epidemiology

Pheochromocytomas can occur at any age, including in childhood, but most often they are detected in the fourth and fifth decades. There is no gender preference. In Western countries the prevalence of pheochromocytoma is estimated between 1:6500 and 1:2500, with an annual incidence of 3 to 8 cases per 1 million per year in the general population, although autopsies show a higher incidence. The pheochromocytoma to paraganglioma ratio is about 0.80 to 0.20. About 35% are familial, and 3% to 50% are malignant, depending on their genetic background.

4. Genetics

There are no lifestyle related risk factors that increase the risk of pheochromocytoma. However, the understanding of the role of genetics has dramatically increased over the last years. Up to 35% of pheochromocytomas are hereditary, and a significant number of patients with apparently sporadic tumors carry a germline mutation. Thus, gene mutations are the largest risk factor involved in the development of pheochromocytoma.

At present, at least 14 well-known susceptibility genes have been discovered that fall into two categories: major susceptibility genes and minor susceptibility genes. Major susceptibility genes represent about 85% to 90% of all hereditary tumors: the VHL gene, which causes von Hippel–Lindau syndrome; the RET gene, for multiple endocrine neoplasia (MEN) types 2A and 2B; the NF1 gene in neurofibromatosis type 1; and the SDHB and SDHD genes in familial paraganglioma syndromes. Minor susceptibility genes include SDHA, SDHC, SDH5/SDHAF2, MAX, TMEM127, EGLN1/PHD2, IDH1, KIF1Bβ and HIF2α, which represent 10% to 15% of hereditary tumors. The list of susceptibility genes is constantly growing, with recently reported genes having a very low incidence; therefore, some of their characteristics have not yet been fully elucidated. We expect more genes to be reported in connection with familial pheochromocytoma but their relevance must be confirmed. The characteristics of hereditary tumors are described in Table 1.

Pheochromocytomas can occur as part of several syndromes, which are associated with additional clinical conditions (Box 1). Latest described Pacak-Zhuang syndrome connects novel mutations in the gene-encoding hypoxia-inducible factor 2α (Hif-2α) with paraganglioma, polycythemia, and somatostatinoma. Other rare syndromes that include pheochromocytomas are Carney triad and Carney–Stratakis syndrome, which are characterized by gastrointestinal stromal tumors and paragangliomas in SDHB and SDHD carriers. It is well established that renal cell carcinomas are also related to SDHB, SDHC, and SDHD gene mutations.

Genetic counseling is recommended for all patients with pheochromocytoma, but it would be neither appropriate nor cost-effective to test for each disease-causing gene in every patient with a pheochromocytoma. An algorithm that takes family history, clinical characteristics, and biochemical phenotype into consideration is shown in Figure 2. In cases of confirmation of a hereditary disorder, one should offer specific genetic tests and genetic

counseling to the patient's family members. Disease screening should be offered to presymptomatic relatives who have a diagnosed mutation, especially because familial syndromes are also associated with other types of tumors and early diagnosis improves the prognosis of these patients.

Presymptomatic genetic testing in minors can raise ethical and legal issues, partly owing to the potential emotional impact of the results and the difficulty of obtaining individual informed consent for the testing of minors. To address these issues, the criteria for proper genetic testing should include several steps (Box 2).

5. Clinical Manifestations

The signs and symptoms of pheochromocytoma are mostly the result of the hemodynamic and metabolic actions of the often inconsistent and disorderly secreted catecholamines on α- and β-adrenoceptors. Most symptoms are nonspecific, including dyspnea, nausea, weakness, weight loss, visual disturbances, arrhythmias, and mental problems, but when a triad of headaches, palpitations, and sweating is accompanied by hypertension, pheochromocytoma should immediately be suspected. The typical episodic symptoms of catecholamine secretion seen in patients (e.g., palpitations, sweating, and headache) may be caused by manipulation of the tumor, endoscopy, anesthesia, ingestion of food or beverages that contain tyramine, and certain medications. However, very often these symptoms occur spontaneously. Psychological stress does not seem to provoke a hypertensive crisis. Many patients have no symptoms or only minor ones. The diagnosis can therefore be easily missed. This is especially true in elderly patients.

Pheochromocytoma can also be discovered during preventive screening, as a result of signs and symptoms related to a mass effect of the tumor, and as incidental findings during imaging studies.

The primary clinical indicators for the diagnosis of pheochromocytoma are summarized in Box 3.

6. Differential Diagnosis

Pheochromocytoma is often referred to as “the great mimic,” because it has signs and symptoms that are common in numerous other clinical conditions. As a result, this often leads to the misdiagnosis of pheochromocytoma. Consideration should be given to other conditions that are associated with sympathomedullary activation (e.g., hyperadrenergic hypertension, renovascular hypertension, panic disorders), because they mimic pheochromocytoma most closely. This overlap can be excluded by a normal response to the clonidine suppression test.

7. Biochemical Diagnosis

Missing a pheochromocytoma can have a fatal outcome. Therefore, tests with high sensitivity are needed to safely exclude a pheochromocytoma without using expensive and unnecessary biochemical follow-up or imaging studies.

Pheochromocytomas can secrete all, none, or any combination of catecholamines (epinephrine, norepinephrine, dopamine). After multiple studies at the National Institutes of Health (NIH), measurement of plasma free metanephrines (the O-methylated metabolites of parent catecholamines), which represent metabolism of catecholamines, but not their secretion, showed superior combined diagnostic sensitivity (98%) and specificity (92%) over all other tests examined, including urinary and plasma catecholamines, urinary total and fractionated metanephrines, and urinary vanillylmandelic acid (VMA). However, the relative advantage of measuring plasma free metanephrines compared to fractionated urinary metanephrines is small. Therefore, expert recommendations for initial biochemical testing include measurement of urine fractionated or plasma free metanephrines, or both if possible.

The conditions under which blood samples are collected can be crucial to the reliability and interpretations of test results. The optimal circumstances are noted in Box 4. Besides these conditions, numerous foods and medications can cause direct or indirect interference in the measurement of catecholamines and metanephrines. This should be kept in mind when interpreting a positive test result. Tricyclic antidepressants, phenoxybenzamine (Dibenzyline), acetaminophen, monoamine oxidase inhibitors, and other drugs interfere

with test results. Tricyclic antidepressants and phenoxybenzamine lead to elevated norepinephrine and normetanephrine levels. Patients with chronic kidney disease, particularly those on dialysis, commonly have elevated plasma metanephrines, even in the absence of pheochromocytoma. Use of liquid chromatography tandem mass spectrometry (LCMS/MS) is the recommended detection method, because it can remove potentially interfering substances. It is also faster, cheaper, and more specific than other techniques.

Besides the initial biochemical tests, which can exclude the disease, follow-up tests are required to establish the diagnosis. This is necessary because although the initial tests are specific, the diagnosis of pheochromocytoma is so rare that there are many false positive results. Options for biochemical follow-up testing are repeated plasma or urinary metanephrine tests, additional sampling for plasma free or urinary fractionated catecholamines, and the clonidine (Catapres) suppression test. Biochemical follow-up testing is not necessary for patients with increases above four times the upper reference limit (URL) of plasma free metanephrines, which are almost always diagnostic for the presence of pheochromocytoma. The previously used glucagon stimulation test should be abandoned, because this test is insufficiently sensitive and can lead to hypertensive complications.

With the increasing proportion of familial tumors, it is important to highlight their different catecholamine profiles. The biochemical profile of a tumor can help guide genetic testing, as reflected in the genetic testing algorithm depicted in Figure 2. Biochemical measurements can also help identify metastatic tumors; a recent study introduced the O-methylated metabolite of dopamine, plasma methoxytyramine, as the most accurate biomarker for discriminating between patients with and without metastases. Several previous studies suggested that increased dopamine could have prognostic significance for metastatic pheochromocytomas, but later methoxytyramine was shown to be a more sensitive bio-marker of a tumor’s dopamine production than either plasma or urinary dopamine.

Based on these findings, an algorithm for biochemical diagnosis was designed and is shown in Figure 3.

8. Localization of pheochromocytoma

Imaging studies to locate pheochromocytoma should be initiated once there is clear biochemical evidence. For optimal results, anatomic imaging studies such as CT or MRI should be combined with high-specificity functional imaging studies. Computed tomography (CT) rather than magnetic resonance imaging (MRI) was suggested as the first-choice imaging modality because of its excellent spatial resolution of the thorax, abdomen, and pelvis. Use of MRI (T2-weighted) is recommended in patients with metastatic pheochromocytoma, for detection of skull base and neck paragangliomas in patients with surgical clips, in patients with an allergy to CT contrast and for patients in whom radiation exposure should be limited (children, pregnant women, patients with known germline mutations, and those with recent excessive radiation exposure).

Initial imaging should be focused on the adrenals. Negative imaging of the adrenals should be followed by CT or MRI scans of the abdomen and pelvis, where paragangliomas are most commonly located. If these scans are negative, chest and neck images should be obtained. Ultrasound is not recommended to localize pheochromocytoma. Exceptions include children and pregnant women when MRI is not available.

After anatomic imaging, which lacks the specificity to indisputably identify a mass as a pheochromocytoma, functional imaging methods can confirm a tumor as a pheochromocytoma. Functional imaging also detects most cases of metastatic and multifocal disease. They include 123I-MIBG scintigraphy, PET, and somatostatin receptor scintigraphy (Octreoscan), which is not recommended for hereditary tumors. PET scanning is preferred for comprehensive localization of metastatic disease. The most commonly used radiopharmaceuticals in PET scanning are 18F-fluorodopamine (18F-FDA), 18F-3,4-dihydroxyphenylalanine (18F-FDOPA), and 18F-fluorodeoxyglucose (18F-FDG) and recently introduced 68Ga-DOTATATE; different circumstances require different radiopharmaceuticals (Figure 4). The 68Ga-DOTATATE or 18F-FDOPA PET scan is recommended as the initial imaging modality for head and neck paragangliomas, and the 18F-FDG or 68Ga-DOTATATE PET scan is recommended for metastatic SDHB-related pheochromocytomas. The use of 123I-MIBG scintigraphy in patients with known metastatic pheochromocytoma should be limited to the evaluation of whether a patient qualifies for 131I-MIBG treatment. A combined PET–MRI scan has been introduced and might represent a novel advantageous imaging modality. The algorithm described in Figure 4

provides the basis for diagnostic localization of pheochromocytoma.

If all tests return negative, it is advised to repeat noninvasive localization after 2 to 6 months.

9. Treatment

The optimal therapy for a pheochromocytoma is prompt surgical removal of the tumor, because an unresected tumor represents a time bomb waiting to explode with a lethal hypertensive crisis. In patients with extensive or metastatic disease, surgery can reduce the hormone secretion and prevent critical anatomic complications, such as urinary tract or cord compression or cardiac obstruction. Safe surgical removal requires the efforts of a team made up of an internist, an anesthesiologist, and a surgeon, preferably in a center experienced with this demanding surgery.

10. Medical Therapy and Preparation for Surgery

The goal of preoperative medical treatment is to control hypertension, maintain stable blood pressure during surgery, minimize adverse effects during anesthesia, and reduce other clinical signs and symptoms caused by high plasma levels of catecholamines.

As soon as the diagnosis is made, blood pressure should be adequately treated for at least 2 weeks before the operation. With satisfactory pretreatment, perioperative mortality has fallen to less than 3% α-adrenergic blockade is the basis of medical management and preoperative preparation. The most commonly used nonselective α-adrenoceptor blocker is phenoxybenzamine, which is also used for nonhypertensive patients. Other possibilities include α-blocking agents such as prazosin, terazosin, and doxazosin. Though these have a shorter duration of action and more often cause hypotension when initially administered for preoperative blood pressure control, postoperative hypotension is more often seen with phenoxybenzamine. In addition to α-blockers, one can use β-blockers (especially when cardiac tachy- and other arrhythmias occur) and calcium channel blockers such as nicardipine. α-Methyl-L-tyrosine and metyrosine has limited use as a premedication. Diuretics should be avoided.

β-blockers should never be used until α-adrenoceptor blockers have been administered for at least 2 to 3 days, because this can result in severe hypertensive crisis in patients with pheochromocytoma, which is believed to result from inhibition of β2-adrenoceptor mediated vasodilation (in the presence of catecholamine stimulation of incomplete α-adrenoceptor blockage). It might be presumed that cardioselective β1-adrenoceptor blocking drugs might be administered without adverse effect. Indeed, almost all adverse reactions to β-blockers in pheochromocytoma patients have involved nonselective β-blockers. Therefore, cardioselective β-blockers (such as atenolol, esmolol, and metoprolol) are favored over nonselective blockers for the management of patients with pheochromocytoma. Nevertheless, because of incomplete specificity and likelihood of some actions on β2-adrenoceptors, even β-blockers deemed to be cardioselective should only be administered to patients with pheochromocytoma once there is adequate control of blood pressure by α-adrenoceptor blockade or other means.

Patients can be recommended a salt- and fluid-rich diet. A proposed algorithm for preoperative treatment is given in Figure 5.

11. Operative and Postoperative Management

After extensive preoperative preparation, surgery should be performed by an experienced surgical and anesthesiology team.

To ensure adequate preoperative preparation, several criteria have been proposed. First, targeted blood pressure should be below 140/90 mm Hg for at least 24 hours. Orthostatic hypotension should be present, but not below 80/45 mm Hg. In some cases, Doppler or conventional echocardiography are indicated in addition to ECG to detect the presence of cardiomyopathy or coronary artery disease. In patients with a large left adrenal pheochromocytoma, splenectomy is likely; therefore, vaccinations against Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis should be given preoperatively.

An experienced anesthesiologist should be aware of potential catecholamine release either as a side effect of the drugs used or as a result of tumor manipulation during the surgery.

A minimally invasive approach is the accepted standard for small, noninvasive, nonmetastatic pheochromocytomas and retroperitoneal paragangliomas, because of its significant postoperative benefits. Locoregional invasion is difficult to establish preoperatively; therefore, it has been recommended that potentially invasive tumors should be initially explored by laparoscopy or retroperitoneoscopy followed by conversion to open surgery in cases of critical adhesion. To prevent permanent glucocorticoid deficiency in patients with bilateral pheochromocytomas, adrenal cortexsparing surgery is advocated. There are multiple potential hazardous events and situations during surgery, including anesthesia induction, tumor manipulation, hypotension, and hypoglycemia. The treatment of hypotension with pressor agents is not recommended, especially when long-acting β-blockers or metyrosine have been used; these paralyze the vascular bed in a dilated state. Instead, volume replacement is the treatment of choice.

Postoperative hypertension can indicate incomplete tumor resection. However, during the first 24 hours after surgery, hypertension is most likely attributed to pain, volume overload, or autonomic instability, all of which are treated symptomatically. If hypertension persists, any attempts to collect specimens for biochemical evidence of an incompletely resected tumor should be delayed for at least 5 to 7 days after surgery to ensure that the large increases in both plasma and urinary catecholamines produced by surgery have dissipated.

Close monitoring of blood glucose in the postoperative period is recommended, because its level can be decreased due to decreased glucose production and increased glucose utilization in the absence of the previous catecholamine excess and persistence of α-adrenoceptor blockers. If the patient is hypotensive, hemorrhage should be excluded first; however, the most likely cause of hypotension is the prolonged effect of the α-adrenoceptor blockers in the presence of reduced plasma catecholamine levels.

12. Hypertensive Crisis

The most dangerous complication of pheochromocytoma is the occurrence of a hypertensive crisis. Hypertensive crisis can manifest as a severe headache, visual disturbances, acute myocardial infarction, congestive heart failure, or a cerebrovascular accident. It is treated with an intravenous bolus of 5 mg phentolamine, a reversible nonselective α-adrenergic antagonist. Phentolamine has a very short half-life, and therefore

the same dose can be repeated every 2 minutes until hypertension is adequately controlled. Phentolamine can also be given as a continuous infusion. Continuous intravenous infusion of sodium nitroprusside or, in some cases, oral or sublingual nifedipine, can also be given to control hypertension.

13. Malignant Pheochromocytoma

Malignant pheochromocytoma is established only by the presence of metastases at sites where chromaffin cells are normally absent. Paragangliomas are malignant more commonly than pheochromocytomas (25% vs. 7%).

Pheochromocytoma metastasizes via hematogenous or lymphatic routes, and the most common metastatic sites are lymph nodes, bones, lung, and liver. About one half of malignant tumors are found at original presentation, and the other half develop at a median interval of 5.6 years, but they can be delayed up to 24 years. Based on the localization of the metastatic lesions, there are short-term and long-term survivors.

Up to 50% of malignant pheochromocytomas develop because of a germline mutation. SDHB mutations with the presence of pheochromocytoma represent about 70% or even more of the risk of malignancy (both in children and adults). Currently, there are several other independent factors of malignancy, including extra-adrenal localization (paragangliomas), the size of the primary tumor (larger than 5 cm), and high methoxytyramine level. Owing to the substantial amounts of methoxytyramine produced by a significant portion of metastatic pheochromocytomas, this measurement should also offer utility in patient management as a surrogate biomarker to assess tumor burden, disease progression, and response to treatment.

Malignant disease is often complicated by clinical manifestations of catecholamine excess and is invariably fatal. The 5-year survival probability after the diagnosis of the first metastasis is reported to be 36% in SDHB carriers and 67% in the absence of this mutation.

Successful management of malignant pheochromocytoma requires a multidisciplinary approach, where pharmacologic treatment, targeted radiotherapy, chemotherapy, and surgery can all play a part. While external-beam radiation has been used for inoperable tumors or for symptom palliation, especially in the treatment of bone lesions, surgical

debulking is considered the mainstay of palliative treatment. About 30% of patients receiving CVD (cyclophosphamide, vincristine, and dacarbazine) exhibit clinical benefits; this number is much higher in patients with SDHB-related malignant tumors (about 70%–80%). Limited documented experience with other chemotherapeutic regimes is available. Somatostatin analogues can be used as an alternative option (for example, DOTATATE). Nowadays, a lot is expected from the novel molecular targeted therapies. In fact, some therapies have already been tested in clinical settings with new possible targets emerging, especially in HIF genes, the mTOR pathway and Hsp90. Individualized treatment should be performed with the intention to cure limited disease and achieve palliation for advanced disease. Figure 6 shows a proposed algorithm for the treatment of metastatic pheochromocytoma.

14. Prognosis and Monitoring

The long-term survival of patients after successful removal of a benign pheochromocytoma is essentially the same as that of age-adjusted normal subjects. Findings from a large study with a long term follow-up showed a recurrence rate of 17%, with half the patients showing signs of malignant disease. Recurrences occur more often in patients with extra-adrenal disease and in patients with a hereditary disorder. At least 25% of patients remain hypertensive after treatment, but this is usually easily controlled with medication.

Clinical follow-up should be lifelong for all patients, but especially in those with an underlying hereditary disorder. The frequency of checkups, once a year or more often, and the kind of diagnostic measurements, only biochemical tests or also imaging studies, should depend on the characteristics of the pheochromocytoma. Follow-up must be more intensive in patients with hereditary and malignant pheochromocytoma.

15. Perspectives

Hormone assessment is currently crucial for the diagnosis of PHEO/PGL. However, there are several pitfalls that have to be considered (e.g. daily rhythm, sex/age dependency, technical limitations of assays, drug interactions). Furthermore, normal ranges vary substantially, depending on the method used, so it is essential to interpret test results in the

context of the appropriate reference range as stated in the European Society of Endocrinology Clinical Practice Guideline.

With the outgoing research the guidelines for biochemical testing changes. Recently Publisher work confirmed that measurement of plasma free MN and NMN with LCMS/MS is not affected by use of β-blockers, diuretics and ACE inhibitors. Withdrawal of these drugs prior to the quantification of plasma metanephrines is therefore not necessary. Studied were following drugs: hydrochlorothiazide, chlorotalidone, enalapril, perindopril, lisinopril, ramipril, metoprolol, propranolol, labetalol. Adding to standard blood sampling, assessment of salivary metanephrines might become a novel and clinically useful biochemical screening tool for PHEO/PGL, particularly suitable for children; as well as for periodic screening of patients with PHEO/PGL syndrome family members.

68Ga-DOTATATE (68Ga labeled somatostatine analogue) PET/CT seems to be the near future of PHEO/PGL functional imaging. Its superiority in the localization of sporadic metastatic PHEO/PGL compared to all other functional and anatomical imaging modalities has been already demonstrated and modification of guidelines were suggested. This observation is also valid for head and neck PGL. Apart for use of this novel somatostatine analogue in functional imaging its future use can be also seen in peptide receptor radionuclide therapy, a potential novel treatment option in patients with head and neck PHEO/PGL.

Up to 10% of patients with non-syndromic presentation are carriers of germ-line mutations. Hence, current approach in diagnostic genetic screening may miss patients with an underlying genetic cause. The limited use of genetic screening in the clinical setting is mainly due to the shortcomings of current methodologies. This includes poor cost effectiveness and long analysis times especially for extensive analyses such as genes of interest. The advent of next-generation sequencing methods has the potential to decrease the cost of sequencing and enable all patients with PPGLs to be screened for all relevant loci.

The patient’s genomic sequence will be the most important factor for patient stratification. Genotype-tailored selection of molecular imaging tracers has been already shown to increase sensitivity of such investigations. Currently it has been hypothesized that, among PHEO/PGL patients with metastatic disease, some could respond differently to systemic treatment; however, this remains to be confirmed in prospective randomized trials.

PHEOs/PGLs are usually benign, only about 10% of cases may be malignant. Unfortunately, there is still lack for reliable prognostic makers. Future clinical validation might confirm molecular biomarkers such as the Ki67 labeling index, loss of cell adhesion molecules (CD44) and human telomerase reverse transcriptase expression as useful markers in detecting malignancy in those tumors. As novel potential biomarkers have been evaluated - DNA methylation and microRNA expression profiles. DNA methylation profiling discovered that RDBP (negative elongation factor complex member E) is related to the presence of metastasis in PCC/PGL. Thus, RDBP could be used for stratifying patients according to the risk of developing metastases. Also, Patterson et al. analyzed miRNA expression in benign and malignant pheochromocytoma tumor samples using whole genome microarray profiling and found that miR-483-5p, miR-183, and miR-101 had significantly higher expression in malignant tumors as compared to benign tumors. In addition, these miRNAs could be detected in pheochromocytoma patient serum.

Tumor hypoxia and its main mediators the HIFs regulate many important biological hallmarks of cancer ranging from genetic instability and tumor cell differentiation to metabolic reprogramming and tumor vascularization. Experimental and clinical data from various tumor types suggest that HIFs also regulate metastasis and treatment resistance, which account for the majority of cancer-related deaths. Thus, HIF inhibitors are likely to target multiple important carcinogenetic processes.

Genotype tailored treatment options, follow-up and preventive care are being investigated. Future developments in PHEO/PGL will mainly focus on further identification of driver mechanisms behind both disease initiation and malignant progression.

16. References - Theoretical Part (Authors listed in alphabetical order)

1. Adjalle R, Plouin PF, Pacak K, Lehnert H. Treatment of malignant pheochromocytoma. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 2009; 41:687-696

2. Amar L, Baudin E, Burnichon N, Peyrard S, Silvera S, Bertherat J, Bertagna X, Schlumberger M, Jeunemaitre X, Gimenez-Roqueplo AP, Plouin PF. Succinate dehydrogenase B gene mutations predict survival in patients with malignant pheochromocytomas or paragangliomas. The Journal of clinical endocrinology and metabolism 2007; 92:3822-3828

3. Ayala-Ramirez M, Feng L, Habra MA, Rich T, Dickson PV, Perrier N, Phan A, Waguespack S, Patel S, Jimenez C. Clinical benefits of systemic chemotherapy for patients with metastatic pheochromocytomas or sympathetic extra-adrenal paragangliomas: insights from the largest single-institutional experience. Cancer 2012; 118:2804-2812

4. Bayley JP, Kunst HP, Cascon A, Sampietro ML, Gaal J, Korpershoek E, Hinojar-Gutierrez A, Timmers HJ, Hoefsloot LH, Hermsen MA, Suarez C, Hussain AK, Vriends AH, Hes FJ, Jansen JC, Tops CM, Corssmit EP, de Knijff P, Lenders JW, Cremers CW, Devilee P, Dinjens WN, de Krijger RR, Robledo M. SDHAF2 mutations in familial and sporadic paraganglioma and phaeochromocytoma. Lancet Oncol 2010; 11:366-372

5. Baysal BE. Screening: Correlation of genotype and phenotype in paraganglioma. Nat Rev Endocrinol 2009; 5:594-595

6. Bjorklund P, Pacak K, Crona J. Precision medicine in pheochromocytoma and paraganglioma: current and future concepts. Journal of internal medicine 2016;

7. Eisenhofer G, Lenders JW, Siegert G, Bornstein SR, Friberg P, Milosevic D, Mannelli M, Linehan WM, Adams K, Timmers HJ, Pacak K. Plasma methoxytyramine: a novel biomarker of metastatic pheochromocytoma and paraganglioma in relation to established risk factors of tumour size, location and SDHB mutation status. Eur J Cancer 2012; 48:1739-1749

8. Eisenhofer G, Rivers G, Rosas AL, Quezado Z, Manger WM, Pacak K. Adverse drug reactions in patients with phaeochromocytoma: incidence, prevention and management. Drug Saf 2007; 30:1031-1062

9. Fassnacht M, Arlt W, Bancos I, Dralle H, Newell-Price J, Sahdev A, Tabarin A, Terzolo M, Tsagarakis S, Dekkers OM. Management of adrenal incidentalomas: European Society of Endocrinology Clinical Practice Guideline in collaboration with the European Network for the Study of Adrenal Tumors. European journal of endocrinology / European Federation of Endocrine Societies 2016; 175:G1-G34

10. Fishbein L, Nathanson KL. Pheochromocytoma and paraganglioma: understanding the complexities of the genetic background. Cancer genetics 2012; 205:1-11

11. Gimenez-Roqueplo AP, Dahia PL, Robledo M. An update on the genetics of paraganglioma, pheochromocytoma, and associated hereditary syndromes. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 2012; 44:328-333

12. Gimm O, DeMicco C, Perren A, Giammarile F, Walz MK, Brunaud L. Malignant pheochromocytomas and paragangliomas: a diagnostic challenge. Langenbeck's archives of surgery / Deutsche Gesellschaft fur Chirurgie 2012; 397:155-177

13. Janssen I, Chen CC, Millo CM, Ling A, Taieb D, Lin FI, Adams KT, Wolf KI, Herscovitch P, Fojo AT, Buchmann I, Kebebew E, Pacak K. PET/CT comparing 68Ga-DOTATATE and other radiopharmaceuticals and in comparison with CT/MRI for the localization of sporadic metastatic pheochromocytoma and paraganglioma. European journal of nuclear medicine and molecular imaging 2016;

14. Janssen I, Chen CC, Taieb D, Patronas NJ, Millo CM, Adams KT, Nambuba J, Herscovitch P, Sadowski SM, Fojo AT, Buchmann I, Kebebew E, Pacak K. 68Ga-DOTATATE PET/CT in the Localization of Head and Neck Paragangliomas Compared with Other Functional Imaging Modalities and CT/MRI. Journal of nuclear medicine : official publication, Society of Nuclear Medicine 2016; 57:186-191

15. Karasek D, Shah U, Frysak Z, Stratakis C, Pacak K. An update on the genetics of pheochromocytoma. J Hum Hypertens 2012;

16. King KS, Prodanov T, Kantorovich V, Fojo T, Hewitt JK, Zacharin M, Wesley R, Lodish M, Raygada M, Gimenez-Roqueplo AP, McCormack S, Eisenhofer G, Milosevic D, Kebebew E, Stratakis CA, Pacak K. Metastatic pheochromocytoma/paraganglioma related to primary tumor development in childhood or adolescence: significant link to

SDHB mutations. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2011; 29:4137-4142

17. Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, Murad MH, Naruse M, Pacak K, Young WF, Jr. Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline. The Journal of clinical endocrinology and metabolism 2014; 99:1915-1942

18. Lenders JW, Eisenhofer G, Mannelli M, Pacak K. Phaeochromocytoma. Lancet 2005; 366:665-675

19. Lenders JW, Pacak K, Huynh TT, Sharabi Y, Mannelli M, Bratslavsky G, Goldstein DS, Bornstein SR, Eisenhofer G. Low sensitivity of glucagon provocative testing for diagnosis of pheochromocytoma. The Journal of clinical endocrinology and metabolism 2010; 95:238-245

20. Mannelli M, Castellano M, Schiavi F, Filetti S, Giacche M, Mori L, Pignataro V, Bernini G, Giache V, Bacca A, Biondi B, Corona G, Di Trapani G, Grossrubatscher E, Reimondo G, Arnaldi G, Giacchetti G, Veglio F, Loli P, Colao A, Ambrosio MR, Terzolo M, Letizia C, Ercolino T, Opocher G. Clinically guided genetic screening in a large cohort of italian patients with pheochromocytomas and/or functional or nonfunctional paragangliomas. The Journal of clinical endocrinology and metabolism 2009; 94:1541-1547

21. Mannelli M, Dralle H, Lenders JW. Perioperative management of pheochromocytoma/paraganglioma: is there a state of the art? Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 2012; 44:373-378

22. Martucci VL, Pacak K. Pheochromocytoma and paraganglioma: diagnosis, genetics, management, and treatment. Current problems in cancer 2014; 38:7-41

23. Matro J, Giubellino A, Pacak K. Current and future therapeutic approaches for metastatic pheochromocytoma and paraganglioma: focus on SDHB tumors. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 2013; 45:147-153

24. Neary NM, King KS, Pacak K. Drugs and pheochromocytoma--don't be fooled by every elevated metanephrine. The New England journal of medicine 2011; 364:2268-2270

25. 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. Germ-line mutations in nonsyndromic pheochromocytoma. The New England journal of medicine 2002; 346:1459-1466

26. Osinga TE, Kema IP, Kerstens MN, de Jong WH, van Faassen M, Dullaart RP, Links TP, van der Horst-Schrivers AN. No influence of antihypertensive agents on plasma free metanephrines. Clinical biochemistry 2016;

27. Osinga TE, van der Horst-Schrivers AN, van Faassen M, Kerstens MN, Dullaart RP, Pacak K, Links TP, Kema IP. Mass spectrometric quantification of salivary metanephrines-A study in healthy subjects. Clinical biochemistry 2016;

28. Pacak K. Preoperative management of the pheochromocytoma patient. The Journal of clinical endocrinology and metabolism 2007; 92:4069-4079

29. Pacak K, Eisenhofer G, Goldstein DS. Functional imaging of endocrine tumors: role of positron emission tomography. Endocr Rev 2004; 25:568-580

30. Pasini B, McWhinney SR, Bei T, Matyakhina L, Stergiopoulos S, Muchow M, Boikos SA, Ferrando B, Pacak K, Assie G, Baudin E, Chompret A, Ellison JW, Briere JJ, Rustin P, Gimenez-Roqueplo AP, Eng C, Carney JA, Stratakis CA. Clinical and molecular genetics of patients with the Carney-Stratakis syndrome and germline mutations of the genes coding for the succinate dehydrogenase subunits SDHB, SDHC, and SDHD. Eur J Hum Genet 2008; 16:79-88

31. Patterson E, Webb R, Weisbrod A, Bian B, He M, Zhang L, Holloway AK, Krishna R, Nilubol N, Pacak K, Kebebew E. The microRNA expression changes associated with malignancy and SDHB mutation in pheochromocytoma. Endocrine-related cancer 2012; 19:157-166

32. Pillai S, Gopalan V, Smith RA, Lam AK. Updates on the genetics and the clinical impacts on phaeochromocytoma and paraganglioma in the new era. Critical reviews in oncology/hematology 2016; 100:190-208

33. Renard J, Clerici T, Licker M, Triponez F. Pheochromocytoma and abdominal paraganglioma. Journal of visceral surgery 2011; 148:e409-416

34. Stefanescu AM, Schipor S, Paun DL, Dumitrache C, Badiu C. Salivary Free Catecholamines Metabolites as Possible Biochemical Markers in Pheochromocytoma Diagnosis. Acta Endocrinologica-Bucharest 2011; 7:431-439

35. Taieb D, Timmers HJ, Hindie E, Guillet BA, Neumann HP, Walz MK, Opocher G, de Herder WW, Boedeker CC, de Krijger RR, Chiti A, Al-Nahhas A, Pacak K, Rubello D. EANM 2012 guidelines for radionuclide imaging of phaeochromocytoma and paraganglioma. European journal of nuclear medicine and molecular imaging 2012; 39:1977-1995

36. Timmers HJ, Chen CC, Carrasquillo JA, Whatley M, Ling A, Havekes B, Eisenhofer G, Martiniova L, Adams KT, Pacak K. Comparison of 18F-fluoro-L-DOPA, 18F-fluoro-deoxyglucose, and 18F-fluorodopamine PET and 123I-MIBG scintigraphy in the localization of pheochromocytoma and paraganglioma. The Journal of clinical endocrinology and metabolism 2009; 94:4757-4767

37. Wigerup C, Pahlman S, Bexell D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. Pharmacology & therapeutics 2016;

38. Zhuang Z, Yang C, Lorenzo F, Merino M, Fojo T, Kebebew E, Popovic V, Stratakis CA, Prchal JT, Pacak K. Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia. The New England journal of medicine 2012; 367:922-930

17. **Plots and Figures – full list is available only for printed copy**

Box 1. Main Clinical Features of Syndromes Associated with Pheochromocytoma

von Hippel–Lindau Syndrome

- Type 1 (No Pheochromocytoma)
    - Renal cell cysts and carcinomas; Retinal and CNS hemangioblastomas; Pancreatic neoplasms and cysts; Endolymphatic sac tumors; Epididymal cystadenomas
- Type 2 (with Pheochromocytoma)
    - Type 2A: Retinal and CNS hemangioblastomas
        - Pheochromocytomas; Endolymphatic sac tumors; Epididymal cystadenomas
    - Type 2B: Renal cell cysts and carcinomas
        - Retinal and CNS hemangioblastomas; Pancreatic neoplasms and cysts Pheochromocytomas; Endolymphatic sac tumors; Epididymal cystadenomas
    - Type 2 C: Pheochromocytomas only

Multiple Endocrine Neoplasia Type 2

- Type 2A (medullary thyroid carcinoma)
    - Pheochromocytomas; Hyperparathyroidism; Cutaneous lichen amyloidosis
- Type 2B (medullary thyroid carcinoma)
    - Pheochromocytomas; Multiple neuromas; Marfanoid habitus
- FMTC: familial medullary thyroid carcinoma only

Neurofibromatosis Type 1

- Multiple benign neurofibromas on skin and mucosa; Cafe´ au lait skin spots; Iris Lisch nodules; Learning disabilities; Skeletal abnormalities; Vascular disease; CNS tumors; Malignant peripheral nerve sheath tumors; Pheochromocytomas;

Paraganglioma Syndromes

- Head and neck tumors
    - Carotid-body tumors
    - Vagal, jugular, and tympanic paragangliomas
- Pacak-Zhuang Syndrome,
    - Multiple paragangliomas
    - Multiple somatostatinomas
    - Polythemia

(Adapted from Lenders JW, Eisenhofer G, Mannelli M, Pacak K: Phaeochromocytoma. Lancet 2005;366:665–75.)

Box 2. Criteria for Proper Genetic Testing in Minors

- Decision should be made by both parents after appropriate consultation with a geneticist.
- Parents should be advised about how to inform their child about the hereditary disease and the reason for genetic testing.
- The discussion of the most appropriate time for testing for each child should take into account the potential medical benefits and the minor's schedule (school schedule, birthdays, etc.).
- Periods of medical examinations or hospitalization for the carrier parent should be avoided where possible.

(Adapted from Lahlou-Laforet K, Consoli SM, Jeunemaitre X, Gimenez- Roqueplo AP. Presymptomatic genetic testing in minors at risk of paraganglioma and pheochromocytoma: Our experience of oncogenetic multidisciplinary consultation. Horm Metab Res 2012;44:354–8.)

Box 3. Patients Who Should Be Evaluated for Pheochromocytoma or Paraganglioma

- Anyone with a triad of headaches, sweating, and tachycardia, whether or not the subject has hypertension
- Anyone with a known mutation of one of the susceptibility genes or a family history of pheochromocytoma
- Anyone with an incidental adrenal mass
- Anyone whose blood pressure is poorly responsive to standard therapy
- Anyone who has had hypertension, tachycardia, or arrhythmia inresponse to anesthesia, surgery, or medications known to precipitate symptoms in patients with pheochromocytoma

Box 4. Optimal Conditions for Blood Collection of Plasma-Free Metanephrines or Catecholamines

- Patient is supine for at least 15 minutes before sampling.
- Samples are collected through a previously inserted IV to avoid stress associated with the needle stick.
- Patient has abstained from nicotine and alcohol for at least 12 hours.
- Patient has fasted overnight before blood sampling.

II. Clinical Part

The size of the primary tumor and age at initial diagnosis are independent predictors of the metastatic behavior and survival of patients with SDHB-related pheochromocytoma and paraganglioma: a retrospective cohort study

Funding:

This work was supported by the Intramural Research Program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development of the National Institutes of Health.

Published:

Schovanek J, Martucci V, Wesley R, Fojo T, Del Rivero J, Huynh T, et al. The size of the primary tumor and age at initial diagnosis are independent predictors of the metastatic behavior and survival of patients with SDHB-related pheochromocytoma and paraganglioma: a retrospective cohort study. BMC cancer. 2014;14(1):523.

Impact Factor 2014: 3.362

Awards:

- 2014 Publishing Award for Young Authors, First prize, Czech Society of Internal Medicine
- 2014 Dean´s Student Publishing Award, Faculty of Medicine and Dentistry, Palacký University Olomouc

1) Commentary

First idea to conduct this study came from my expert tutor prof. Pacak. The original idea was to look more deeply into the specific group of patients harboring mutation in SDHB gene. It has been known for a long time that mutations in succinate dehydrogenase subunit B (SDHB), first described by the pioneering work of Astuti et al. in 2001, have been linked to more aggressive tumor behavior, presenting with a higher metastatic rate than other PHEOs/PGL. Eisenhofer and colleagues have described an increase in the likelihood of metastases in larger PHEOs and PGLs. While SDHB mutations are considered powerful predictors of malignancy, it is unclear why SDHB-related PHEOs/PGLs in particular are more aggressive, often metastatic, and ultimately fatal, even though some other hereditary PHEOs/PGLs. It has been shown that SDHB-related PHEOs/PGLs are most commonly extraadrenal and larger at first presentation, with a characteristic noradrenergic and/or dopaminergic biochemical phenotype, as well as much lower catecholamine tumor concentrations than any other sporadic or hereditary PHEOs/PGLs. As a result of this lower catecholamine content, SDHB patients may initially present with only mild clinical symptoms that do not become worrisome until a sufficient amount of catecholamines is released, often in cases of already large primary tumors.

In the present study, we initially used receiver operating characteristic (ROC) curves to establish cut-off sizes for evaluation of the development of metastases and patient survival.

We hypothesized that even in the presence of an SDHB mutation, smaller tumors would have a statistically significantly lower metastatic potential and longer patient survival than larger tumors. Subsequently we created Cox regression models aiming to establish whether those parameters could also be considered as independent predictors of PHEO/PGL metastatic behavior and patient outcome. The effect of having a specific SDHB gene mutation, adrenal or extra-adrenal tumor location, and their occurrence in males or females were also analyzed statistically. Finally, survival and metastatic potential parameters, including the presence of synchronous and metachronous metastases, were evaluated for both 5-year and overall survival.

2) Introduction

According to the 2004 WHO classification of tumors, pheochromocytomas (PHEOs) arise from chromaffin cells of neural crest origin in the adrenal medulla. Closely related paragangliomas (PGLs) arise from the cells of sympathetic or parasympathetic paraganglia (1). These tumors synthesize catecholamines that are metabolized to metanephrines, which are preferentially used in the biochemical diagnosis of these tumors (2).

Mutations in succinate dehydrogenase subunit B (SDHB), first described by the pioneering work of Astuti et al. in 2001 (3), have been linked to more aggressive tumor behavior, presenting with a higher metastatic rate than other PHEOs/PGLs (3-7). The rate of metastasis of SDHB-related PHEOs/PGLs has been reported to be between 34% (8) and 71% (9), with a 5-year survival rate of 36% after the diagnosis of metastasis (5). In addition, regardless of SDHB mutation status, tumor size has also been shown to be related to developing metastatic disease. Eisenhofer and colleagues described an increase in the likelihood of metastases in PHEOs from less than 6% for tumors smaller than 5 cm to over 50% in tumors larger than 10 cm; for PGLs, the rate of malignancy increases to over 80% for tumors larger than 9 cm (10).

While SDHB mutations are considered powerful predictors of malignancy, it is unclear why only SDHB-related PHEOs/PGLs are more aggressive, often metastatic, and ultimately fatal, even though some other hereditary tumors are also pseudohypoxic. Nevertheless, some unique insights into the presentation and pathogenesis of these tumors have been published recently by Eisenhofer et al (10-11) and Loriot et al. (12). These studies either confirmed or first showed that SDHB-related PHEOs/PGLs are most commonly extra-adrenal and larger at first presentation, with a characteristic noradrenergic and/or dopaminergic biochemical phenotype, as well as much lower catecholamine tumor concentrations than any other sporadic or hereditary PHEOs/PGLs.

Of the unique SDHB-related PHEO/PGL characteristics described above, extra-adrenal location, age of initial presentation, size of the primary tumor, and elevated methoxytyramine levels were introduced and confirmed as risk factors for the metastatic behavior of PHEOs/PGLs (13-16). Therefore, it has been recommended that patients with SDHB-related, large, or extra-adrenal PHEOs/PGLs should have more frequent and lifelong follow-up (17).

In the present study, we aimed to establish whether the size of SDHB-related PHEOs/PGLs could be an important and independent predictor of their metastatic behavior and patient outcome. We hypothesized that smaller SDHB tumors, less than 6 cm, would have a statistically significantly lower metastatic potential and longer survival than larger tumors over 6 cm, independent of the presence of a specific SDHB gene mutation, their adrenal or extra-adrenal location, and their occurrence in males or females. The survival and metastatic potential parameters, including the presence of synchronous and metachronous metastases, were evaluated at both 5 years and overall.

3) Patients

We performed a single center retrospective study, evaluating only patients with SDHB-related PHEOs/PGLs seen for evaluation or treatment at the National Institutes of Health (NIH) Clinical Center, a referral center for these tumors, between 1996 and 2012. All patients provided informed written consent. Pathological, surgical, and imaging study reports were carefully reviewed in order to collect the most accurate information about the patients. The follow-up data and information were collected based on patients' regular follow-up visits at NIH or phone/email contact in the period of 2012-2013.

Metastases were confirmed either by surgery or by anatomical and PHEO/PGL-specific functional imaging studies. When there was evidence of lesions in areas where chromaffin cells are not present, these lesions were considered metastatic. For the purposes of this study, when the metastases were observed together with a recurrent tumor, the tumor was marked as metastatic; recurrences were not evaluated. We used the term synchronous metastases to describe the occurrence of metastases when discovered at the initial diagnosis or within 6 months after the primary tumor diagnosis (15).

For analyses of the effect of the size of the primary tumor for the development of metastases, we used a largest diameter size of 4.5 cm as a cutoff point, the optimal value (which maximizes the sum of the sensitivity and specificity) based on receiver operating characteristic analysis (ROC; AUC = 0.782, sensitivity = 80.5%, specificity = 69%). For survival analysis, we dichotomized the patient cohort using the largest diameter size of 5.5 cm, the optimal cutoff diameter from ROC analysis (AUC = 0.663, sensitivity = 87.0%, specificity = 49.5%); this value divided the patients almost equally. Analyses of the effects

of various parameters on the time to metastases and for survival used Kaplan-Meier curves to graphically represent the results, with group comparisons based on the standard logrank test (to compare 2 groups), the trend version of it (to compare more than 2 groups that are ordered), or stratified versions of these (to adjust for a second parameter, such as PGL vs. PHEO). Survival analyses were reported either for total survival or for survival truncated at 5 years (i.e. anyone whose observation time was longer than 5 years was considered censored at 5 years); the same applies for "survival" analyses of time to metastases.

To analyze the mutual effects of age at diagnosis and the size of the primary tumor on survival, we used Cox regression models, with 5 ordered categories for age and 4 ordered categories for size. Cox regression was also used to estimate the relative hazard rates for the 4 ordered size categories. For an alternative nonparametric, model-free estimate of the probability of death or metastases vs. tumor size, observations were divided into bins and the lowess smoother was applied to the proportions of outcomes in the bins. All survival results used death due to disease as the endpoint. All P-values are two-sided.

4) Results

Patient characteristics and tumor size

One hundred six patients (39 females, 67 males) with SDHB-related tumors from the Eunice Kennedy Shriver National Institute of Child Health & Human Development, NIH PHEO/PGL registry were included in the present study. The number of males in the present study was significantly higher than the number of females ($P = 0.008$), but these two groups did not differ in any of the following parameters: size of the primary tumor ($P = 0.13$); percentage of patients with synchronous metastases ($P = 0.36$); time to metastasis ($P = 0.94$); or overall survival ($P = 0.36$) (Figure 1b).

Eighty-nine patients presented with PGL and 17 with PHEO ($P < 0.001$). The median sizes of the primary PGLs and PHEOs were 6 cm and 8 cm, respectively ($P = 0.028$). The median size of all primary tumors was 6 cm. The survival of patients diagnosed with PHEO appeared slightly worse than of patients diagnosed with PGL, but did not reach statistical significance ($P = 0.099$); the 5-year survival was the same ($P = 0.65$) (Figure 1a).

The median ages at diagnosis with PGL or PHEO were 29 and 31 years, respectively.

The age at diagnosis did not differ for different tumor sizes (Table 1). However, tumors with a smaller diameter were diagnosed significantly more often in the past few years (P = 0.043) (Table 1).

All the patients considered survival failures died due to metastatic disease.

Effect of size on metastasis occurrence

Seventy-seven out of our 106 patients (72.6%) were diagnosed with metastatic disease over the course of their disease. Twenty-eight patients (26.4%) developed metastatic disease at the same time as their primary tumor or within 6 months of initial diagnosis (synchronous metastases); their median age at diagnosis was 31.5 years; the median diameter of primary tumor 7.5 cm. Of the 78 patients who were not diagnosed with synchronous metastases, 49 (46.2%) developed metachronous metastases within the median time of 5 years; the median age at diagnosis was 30 years; the median size of primary tumors 7.0 cm. There was not a significant difference in the ages at diagnosis of patients diagnosed with synchronous metastases and those without synchronous metastases (P = 0.65). For the 29 (27.4%) patients who never developed metastatic disease, the median age at initial diagnosis was 29 years; the median size of the primary tumor was only 3.8 cm. The overall size of the primary tumors was found to be highly statistically different among these 3 reported groups (P < 0.001). Patients with PHEO and PGL did not differ in the time to the development of metastasis (P = 0.54). The probability of a 5-year metastasis-free interval among those without synchronous metastasis was 48.2% for PGL and 55.0% for PHEO.

When we divided the patient cohort using a primary tumor size of 4.5 cm, 20.0% of patients with smaller tumors (< 4.5 cm) and 29.6% of those with larger tumors (≥ 4.5 cm) had synchronous metastases (P = 0.35). The median time to develop metachronous metastases in the group of patients with primary tumors <4.5 cm was 8 years (CI 95%, 3 years to infinity), and for those with larger tumors (≥4.5 cm) it was only 2 years (CI 95%, 1 to 4 years; P = 0.003) (Figure 2c).

Alternatively, the patients were divided into four groups according to the size of the primary tumor (≤ 4 cm, 4-6 cm, 6-9 cm, > 9 cm). This division was both clinically relevant and resulted in groups of almost equal size (32/24/25/25). The percentages with synchronous metastases in these four groups were 22%, 13%, 28%, and 44%, respectively (P = 0.049, exact test for contingency table with ordered columns). The median time to

develop metachronous metastases in the four groups was 8, 4, 3, and 1 years respectively (P = 0.0008), with probabilities of 5-year metastasis-free intervals of 66.2%, 34.6%, 25.1%, and 19.2% (Table 1).

Effect of size on survival time

Based on the 5.5 cm diameter that reflected the optimal cutoff size for the present cohort, patients were divided into two groups: < 5.5 cm and ≥ 5.5 cm. When we analyzed the effect of tumor size on patient survival time between these two groups, we found that patients with primary tumors smaller than 5.5 cm had significantly longer overall survival than patients with larger tumors (P = 0.008, stratified by tumor type) (Figure 2c). When this size division was kept and the two tumor types were analyzed separately, the effect of size was highly significant in PGLs (P = 0.012), but was not significant in PHEOs (P = 0.39). These size-based differences were not statistically significant in the 5-year interval, although for PGLs the effect was already trending toward a difference (P = 0.12).

When the previously introduced alternative division (≤ 4 cm, 4-6 cm, 6-9 cm, > 9 cm) was applied, the effect of size was also significant (trend P = 0.035). The 5-year survival probability for these four groups, listed also for each tumor type separately, is shown in Table 1. Table 1 also shows survival hazard ratios for these four patient groups, with the ≤ 4 cm group serving as a reference group with hazard ratio 1.

Effect of metastases on survival rate

As previously mentioned, 72.6% (77 out of 106) of patients in the present study developed metastatic disease. The 5-year survival probability after the diagnosis of metastases was 75.7% (CI 95%; 63%-84%). The 5-year survival probability for patients who presented with synchronous metastases was 74.5%; for patients without synchronous metastases it was 96.4% (P = 0.006). However, there was no significant difference in 5-year survival once patients were diagnosed with metastatic disease (74.5% for synchronous metastases and 77.0% for metachronous metastases; P = 0.42).

The development of synchronous metastases did not have a significant effect on the survival of patients with the smaller tumors (< 5.5 cm), but it had a highly significant effect on the survival of patients with larger tumors (≥ 5.5 cm). Patients with larger tumors and synchronous metastases had a 5-year survival probability of 65.8%, while patients with the

same size primary tumors, but without synchronous metastases, had a 5-year survival probability of 97.1%. These findings were observed only in PGLs, with this effect not being found in PHEOs, as shown in Table 2.

Furthermore, the 5-year survival probability for PGL patients with synchronous metastases was 73.2%; for those without synchronous metastasis, it was 97.9% (P = 0.0002). Patients with PHEO and synchronous metastases had a 5-year survival probability of 80.0%, and those without synchronous metastases 88.9% (P = 0.56) (Table 2).

Effect of SDHB mutation type

In the present study, patients had a variety of SDHB mutation types: 13 had deletions (PGLs 11/PHEOs 2), 7 had frame-shift mutations (PGLs 4/PHEOs 3), 41 had missense mutations (PGLs 33/PHEOs 8), 24 had nonsense mutations (PGLs 20/PHEOs 4), and 21 had splice site mutations (PGLs 17/PHEOs 4). We did not find any significant differences in tumor size or survival time among different SDHB mutation types (P = 0.74 for size, P = 0.61 for survival time). The smallest tumors were found in the group of patients with frame-shift mutations, the largest tumors in patients with nonsense mutations.

Size and age as independent predictors

In the PGL group, we did not find any interaction between the age at diagnosis and the sizes of the primary tumors (P = 0.70) that affected patient survival. In subsequent evaluation, the size of the primary tumor and the age at initial diagnosis were found to be independent predictors of patient survival (P = 0.007 and P < 0.001, respectively). Patients diagnosed with PGL at a younger age had better survival, as did patients with smaller tumors (< 5.5 cm). Concerning the development of metastases, we did not find any interaction between the age at diagnosis and the primary tumor size (P = 0.11). Furthermore, in the PGL group, age at diagnosis did not predict the development of metastases (P = 0.51), but the size of the primary tumor did (P = 0.003). The time to the development of metastases in PGLs was similar for the different age groups, but patients with larger tumors were more likely to be diagnosed with metastatic disease.

In the PHEO group, we also did not observe any interaction between the age at diagnosis and the size of the primary tumor (P = 0.67) that affected patient survival or the development of metastases (P = 0.75). Age at diagnosis was an independent predictor of patient survival

(P = 0.041) but not of the development of metastases (P = 0.21), as reported for the PGL group. In PHEO, younger patients had better survival, but the same probability of metastatic disease development as older patients. Tumor size was not found to be an independent predictor of patient survival (P = 0.49) or the development of metastases (P = 0.65). The size of the primary PHEO did not affect patient survival or the development of metastases.

5) Discussion

In the present study of 106 patients with pathogenic SDHB germline mutations, we found that the size of the primary tumor is an age-independent predictor of patient survival and metastases development in PGL. In both PHEO and PGL, age at diagnosis was found to be a size-independent predictor of patient survival. Furthermore, the development of synchronous metastases significantly affected 5-year and overall survival in patients with PGL. However, patients with PHEO had worse, though not significant, overall survival than those with PGL (P = 0.099); their survival was not affected by the size of the primary tumor or by synchronous metastases. We did not find a significant difference in metastases development or patient survival between males and females or among specific SDHB mutations.

Studies evaluating exclusively metastatic PHEO/PGL patients have found that about one-third of these patients harbor pathogenic SDHB mutations (4, 9); however, the reported metastatic rate in SDHB-related PHEO/PGL varies dramatically (18). In the present study we found it to be high, as 72.6% of the patients developed metastases over the course of their disease, which is similar to the 71.7% previously reported in the study by Amar et al. (9). Consequently, the presence of an SDHB mutation was found to be an independent predictor of PHEO/PGL malignant behavior (5). Typical metastatic sites of SDHB-related PHEO/PGL include the bones, lungs, lymph nodes, and liver; multiple metastases are also possible (5). Furthermore, the incidence of PHEO/PGL in childhood and adolescence is rare; however, when diagnosed in these age groups, patients have a high probability of having SDHB mutations (71.9%), and the majority (85.2%) develop metastatic disease (19).

The previously reported ratio between synchronous and metachronous metastases in various PHEOs/PGLs was almost equal (51%/49%) (15). In our study with only SDHB-related tumors, we observed predominantly metachronous rather than synchronous

metastases (64%/36%), which could be due to the relatively long follow-up periods for our patients. We did not find a significant difference in the 5-year survival between patients after the diagnosis of synchronous or metachronous metastatic disease.

The observed worse survival of PHEO/PGL patients with SDHB mutations compared to other PHEO/PGL patients might be related to their lower catecholamine content. As a result, SDHB patients initially present with only mild clinical symptoms that do not become worrisome until a sufficient amount of catecholamines is released, even in cases of large primary tumors (10).

Disagreement on the relative survival of patients with PHEO and PGL continues; a previous study, which did not classify the tumors based on genetic background, found overall survival to be significantly shorter in patients with PGLs than with PHEOs (15). A different study, also not considering the genetic background of the disease and including only metastatic PHEO/PGL, reported better survival of PGLs (20), consistent with the present study. Our study included only patients with SDHB-related tumors and found that patients with PGLs tended to have a better survival than patients with PHEOs; however, this difference was not statistically significant. While elucidating other possible differences between SDHB-related PHEO and PGL, we demonstrated that the size of the primary tumor for patients with PHEOs seems to be less important for patient survival than for PGL. We initially attributed this to the selected cut-off (5.5 cm), which was more representative of the size of primary PGLs (and divided them almost evenly) and divided the PHEOs quite unevenly. However, when we used 8 cm as a cutoff, the median size of PHEO primary tumors, there was again no survival difference in PHEOs between those with small vs. large tumors ($P = 0.81$).

Our analysis clearly shows that the size of the primary tumor in PGLs predicts the development of metastatic disease and also affects patient survival, while the age at diagnosis predicts patient survival but not the development of metastases. In contrast, in PHEOs, the age at diagnosis is an important factor for survival, but size is not. This is not in agreement with the previous observation of Zelinka et al. (13), who evaluated a larger sample of metastatic PHEOs, but did not focus on the genetic background of the PHEOs included in that study, which both could explain the presented difference.

The importance of the primary tumor size for patient prognosis in general oncology is

well established, as manifested by the use of the TNM classification (21). Thus, previous studies have already agreed that the size of the primary tumor is an important predictor for patient survival and for the metastatic potential of PHEO/PGL (10, 13, 15). This study extends this knowledge, due to its unique design, to SDHB-related PHEO/PGL.

Initially we determined the optimal cut-off for our study (5.5 cm), separating the smaller and larger tumors; this cut-point was close to the median tumor size of PGLs, by far the larger of the two disease groups. Patients with smaller tumors had significantly better survival than patients with larger tumors. Similarly, we established a size cut-off for the development of metastases as 4.5 cm; patients with smaller tumors developed metastases significantly later than patients with larger tumors. We did not find any difference based on the sex of the patient, but we did observe certain differences between SDHB-related PGLs and PHEOs. PHEOs were significantly larger (also as previously reported (20)), had worse prognoses, and did not show a relationship between tumor size and overall survival. The effect of tumor size on overall survival was highly significant for PGLs, but not present in PHEOs.

Given the status of the NIH PHEO/PGL program as a national and international referral center, our patient population is typically made up of more complicated cases, usually due to patients with underlying genetic backgrounds. Therefore, we were not able to establish a similarly sized cohort of apparently sporadic patients that would allow us to investigate whether these tumors would behave similarly to SDHB-related PHEO/PGL and to elucidate how exactly the presence of an SDHB mutation would affect the development of metastatic disease and patient survival when the size of the primary tumor is considered and compared to other PHEO/PGL types. Since the incidence of SDHB-related PHEOs is very low compared to SDHB-related PGLs, it was very difficult to reliably compare these two groups, and other significant findings might become more apparent if a larger number of PHEOs were available.

The present study showed better survival of younger patients and the independence of age at diagnosis (with tumor size) as a survival predictor. Improved survival of younger patients was already reported in a study by Amar et al., but did not reach significance as an independent predictor (5).

In summary, the present study of patients with SDHB-related PHEO/PGL shows that the

age at the primary diagnosis as well as the size of the primary tumor are two important independent prognostic factors. This data strongly supports our recommendations that all carriers with SDHB mutations should undergo early and regular evaluations to detect tumor(s) at an early stage to achieve the best clinical outcome with regards to their survival.

6) References Clinical Part (Authors listed in *BMJ Journal* order)

1. DeLellis RA 2004 Pathology and genetics of tumours of endocrine organs. Lyon: IARC Press

2. 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

3. Astuti D, Latif F, Dallol A, Dahia PLM, Douglas F, George E, Sk√∂ldberg F, Husebye ES, Eng C, Maher ER 2001 Gene Mutations in the Succinate Dehydrogenase Subunit SDHB Cause Susceptibility to Familial Pheochromocytoma and to Familial Paraganglioma. The American Journal of Human Genetics 69:49-54

4. 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

5. Amar L, Baudin E, Burnichon N, Peyrard S, Silvera S, Bertherat J, Bertagna X, Schlumberger M, Jeunemaitre X, Gimenez-Roqueplo AP, Plouin PF 2007 Succinate dehydrogenase B gene mutations predict survival in patients with malignant pheochromocytomas or paragangliomas. J Clin Endocrinol Metab 92:3822-3828

6. Benn DE, Gimenez-Roqueplo AP, Reilly JR, Bertherat J, Burgess J, Byth K, Croxson M, Dahia PL, Elston M, Gimm O, Henley D, Herman P, Murday V, Niccoli-Sire P, Pasieka JL, Rohmer V, Tucker K, Jeunemaitre X, Marsh DJ, Plouin PF, Robinson BG 2006 Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. J Clin Endocrinol Metab 91:827-836

7. Karasek D, Shah U, Frysak Z, Stratakis C, Pacak K 2013 An update on the genetics of pheochromocytoma. J Hum Hypertens 27:141-147

8. Neumann Hp Fau - Pawlu C, Pawlu C Fau - Peczkowska M, Peczkowska M Fau - Bausch B, Bausch B Fau - McWhinney SR, McWhinney Sr Fau - Muresan M, Muresan M Fau - Buchta M, Buchta M Fau - Franke G, Franke G Fau - Klisch J, Klisch J Fau - Bley TA, Bley Ta Fau - Hoegerle S, Hoegerle S Fau - Boedeker CC, Boedeker Cc Fau - Opocher G, Opocher G Fau - Schipper J, Schipper J Fau - Januszewicz A, Januszewicz A

Fau - Eng C, Eng C 2004 Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations.

9. Amar L Fau - Bertherat J, Bertherat J Fau - Baudin E, Baudin E Fau - Ajzenberg C, Ajzenberg C Fau - Bressac-de Paillerets B, Bressac-de Paillerets B Fau - Chabre O, Chabre O Fau - Chamontin B, Chamontin B Fau - Delemer B, Delemer B Fau - Giraud S, Giraud S Fau - Murat A, Murat A Fau - Niccoli-Sire P, Niccoli-Sire P Fau - Richard S, Richard S Fau - Rohmer V, Rohmer V Fau - Sadoul J-L, Sadoul Jl Fau - Strompf L, Strompf L Fau - Schlumberger M, Schlumberger M Fau - Bertagna X, Bertagna X Fau - Plouin P-F, Plouin Pf Fau - Jeunemaitre X, Jeunemaitre X Fau - Gimenez-Roqueplo A-P, Gimenez-Roqueplo AP 2005 Genetic testing in pheochromocytoma or functional paraganglioma.

10. Eisenhofer G, Lenders JW, Siegert G, Bornstein SR, Friberg P, Milosevic D, Mannelli M, Linehan WM, Adams K, Timmers HJ, Pacak K 2011 Plasma methoxytyramine: A novel biomarker of metastatic pheochromocytoma and paraganglioma in relation to established risk factors of tumour size, location and SDHB mutation status. Eur J Cancer

11. Eisenhofer G, Tischler AS, de Krijger RR 2012 Diagnostic Tests and Biomarkers for Pheochromocytoma and Extra-adrenal Paraganglioma: From Routine Laboratory Methods to Disease Stratification. Endocr Pathol 23:4-14

12. Loriot C, Burnichon N, Gadessaud N, Vescovo L, Amar L, Libe R, Bertherat J, Plouin PF, Jeunemaitre X, Gimenez-Roqueplo AP, Favier J 2012 Epithelial to Mesenchymal Transition Is Activated in Metastatic Pheochromocytomas and Paragangliomas Caused by SDHB Gene Mutations. J Clin Endocrinol Metab

13. Zelinka T, Musil Z, Duskova J, Burton D, Merino MJ, Milosevic D, Widimsky J, Jr., Pacak K 2011 Metastatic pheochromocytoma: does the size and age matter? Eur J Clin Invest 41:1121-1128

14. Eisenhofer G, Lenders JW, Timmers H, Mannelli M, Grebe SK, Hofbauer LC, Bornstein SR, Tiebel O, Adams K, Bratslavsky G, Linehan WM, Pacak K 2011 Measurements of plasma methoxytyramine, normetanephrine, and metanephrine as discriminators of different hereditary forms of pheochromocytoma. Clin Chem 57:411-420

15. Ayala-Ramirez M, Feng L, Johnson MM, Ejaz S, Habra MA, Rich T, Busaidy N, Cote GJ, Perrier N, Phan A, Patel S, Waguespack S, Jimenez C 2011 Clinical risk factors for

malignancy and overall survival in patients with pheochromocytomas and sympathetic paragangliomas: primary tumor size and primary tumor location as prognostic indicators. J Clin Endocrinol Metab 96:717-725

16. O'Riordain DS, Young WF, Jr., Grant CS, Carney JA, van Heerden JA 1996 Clinical spectrum and outcome of functional extraadrenal paraganglioma. World J Surg 20:916-921; discussion 922

17. 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. October 2005. Nat Clin Pract Endocrinol Metab 3:92-102

18. Ricketts CJ, Forman JR, Rattenberry E, Bradshaw N, Lalloo F, Izatt L, Cole TR, Armstrong R, Kumar VK, Morrison PJ, Atkinson AB, Douglas F, Ball SG, Cook J, Srirangalingam U, Killick P, Kirby G, Aylwin S, Woodward ER, Evans DG, Hodgson SV, Murday V, Chew SL, Connell JM, Blundell TL, Macdonald F, Maher ER 2010 Tumor risks and genotype-phenotype-proteotype analysis in 358 patients with germline mutations in SDHB and SDHD. Hum Mutat 31:41-51

19. King KS, Prodanov T, Kantorovich V, Fojo T, Hewitt JK, Zacharin M, Wesley R, Lodish M, Raygada M, Gimenez-Roqueplo AP, McCormack S, Eisenhofer G, Milosevic D, Kebebew E, Stratakis CA, Pacak K 2011 Metastatic pheochromocytoma/paraganglioma related to primary tumor development in childhood or adolescence: significant link to SDHB mutations. J Clin Oncol 29:4137-4142

20. Goffredo P, Sosa JA, Roman SA 2013 Malignant pheochromocytoma and paraganglioma: A population level analysis of long-term survival over two decades. J Surg Oncol 107:659-664

21. Sobin LH, Gospodarowicz MK, Wittekind C, International Union against C 2010 TNM classification of malignant tumours. Chichester, West Sussex, UK; Hoboken, NJ: Wiley-Blackwell

7) Plots and Figures

Table 1: Comparison of the 4 tumor size groups.

	Type of tumor	Size of primary tumor				P-value
		<= 4 cm	4 - 6 cm	6 - 9 cm	> 9 cm	
No. of patients by tumor size group [%]	ALL	32 [30]	24 [23]	25 [24]	25 [24]	**0.034 (PGL vs. PHEO)**
	PGL	31 [35]	19 [21]	20 [22]	19 [21]	
	PHEO	1 [6]	5 [29]	5 [29]	6 [35]	
Probability of 5-year survival [%]	ALL	94.1	95	83.4	88	**0.16**
	PGL	93.8	93.8	85.0	89.5	**0.26**
	PHEO	100	100	75.0	83.3	**0.39**
Years to death (median)	ALL	>55	>25	12	20	**0.035**
	PGL	>55	>25	12	20	**0.030**
	PHEO	>5	9	8	17	**0.58**
Survival Hazard Ratio	ALL	1	4.6	12.21	5.82	
No. of deceased patients [%]	ALL	1 [3.13]	6 [25]	10 [40]	6 [24]	
Median age at diagnosis	ALL	32	25	30	31	**0.44**
Median year of diagnosis	ALL	2007	2003	2004	2003	**0.043**
Years to metastases (median)	ALL	8	4	3	1	**0.0008**
Probability of 5-year "metastases-free interval" [%]	ALL	66.2	34.6	25.1	19.2	**0.0002**
Probability of 10-year "metastases-free interval" [%]	ALL	34.0	12.4	16.8	19.2	**0.004**

Table 2: 5-year survival probability of patients with tumors by tumor type (PHEO vs. PGL) and tumor size based on the presence of synchronous metastasis.

	Without synchronous metastases (n=78)	Synchronous metastases (n=28)	5-year/overall survival p-value
All tumors by type			
PGL (n=89)	97.9% (n=66)	73.2% (n=23)	0.0002/0.001
PHEO (n=17)	88.9% (n=12)	80.0% (n=5)	0.56/0.94
5-year/overall survival p-value	0.19/0.012	0.79/0.89	---
All tumors by size			
<5.5 cm (n=44)	95.2% (n=37)	100% (n=7)	0.63/0.41
>=5.5 cm (n=62)	97.1% (n=41)	65.8% (n=21)	0.0003/0.0028
5-year/overall survival p-value	0.71/0.18	0.09/0.021	---
PGLs by size			
<5.5 cm (n = 41)	94.7% (n=34)	100% (n=7)	0.61/0.40
>=5.5 cm (n=48)	100% (n=32)	60.9% (n=16)	0.0001/0.0002
5-year/overall survival p-value	0.22/0.57	0.068/0.014	---
PHEOs by size			
<5.5 cm (n=3)	100% (n=3)	N/A	N/A
>=5.5 cm (n=14)	85.7% (n=9)	80.0% (n=5)	0.68/0.86
5-year/overall survival p-value	0.59/0.38	N/A	---

Figure 1: Survival and development of metastasis stratified by tumor type (panel A), by gender (panel B).

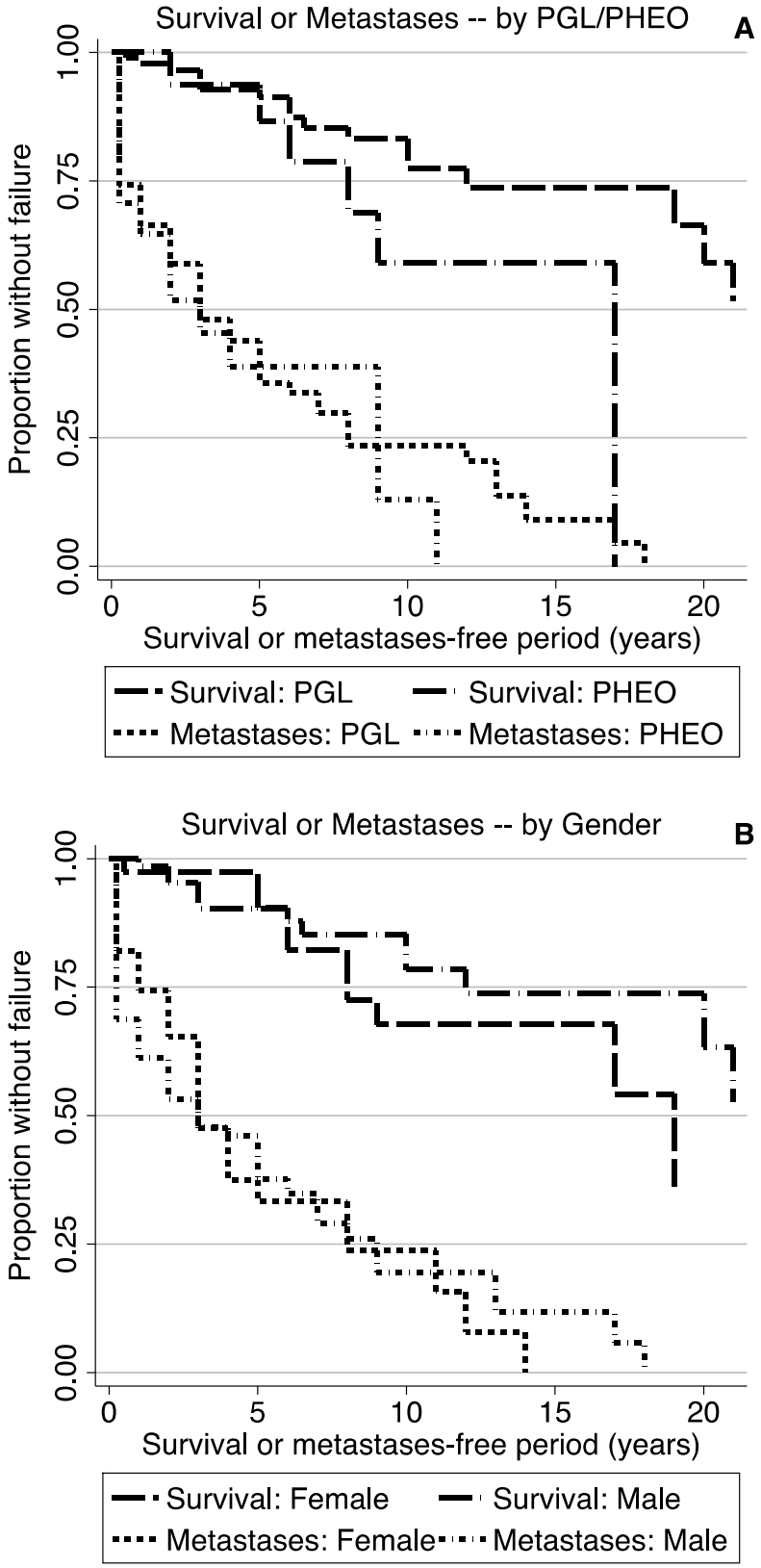
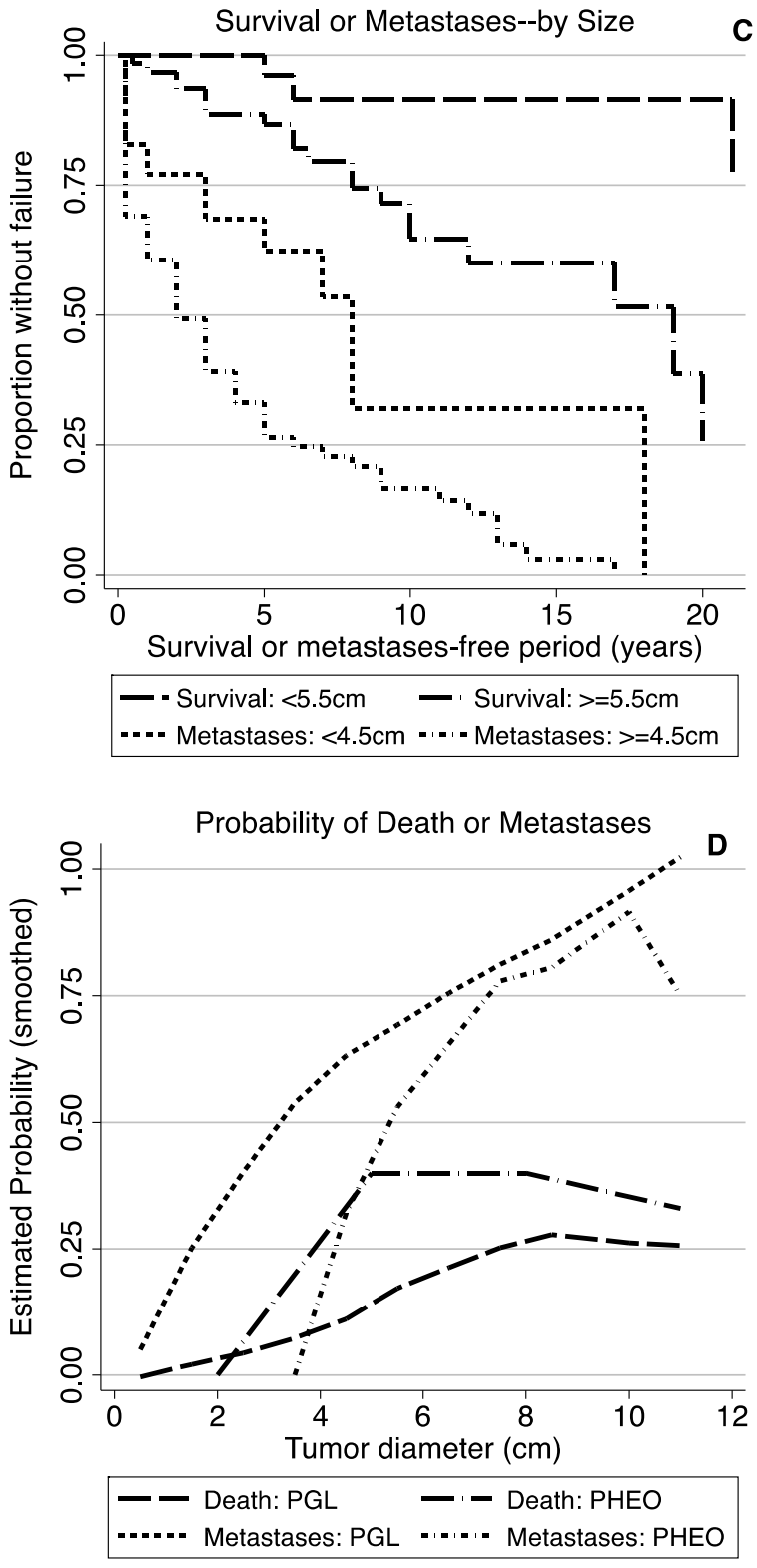


Figure 2: Survival and development of metastasis stratified by primary tumor size (panel A); Survival and development of metastasis based on linearly increasing primary tumor size (panel B).



Pdf version of the published article

III. Experimental Part

Inhibitory effect of the non-camptothecin topoisomerase I inhibitor LMP-400 on female mice models and human pheochromocytoma cells

Funding:

This work was supported by the Intramural Research Program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development of the National Institutes of Health.

Acknowledgements:

James Doroshow, National Cancer Institute, Bethesda, MD, USA

Division of Cancer Treatment and Diagnosis, National Cancer Institute for providing the LMP-400

Published:

Schovanek J, Bullova P, Tayem Y, Giubellino A, Wesley R, Lendvai N, et al. Inhibitory effect of the non-camptothecin topoisomerase I inhibitor LMP-400 on female mice models and human pheochromocytoma cells. Endocrinology. 2015 Aug 12:en20151476.

Impact Factor 2015: 4.159

Awards:

- 2015 Publishing Award for Young Authors, Second prize, Czech Society of Internal Medicine
- 2015 Dean´s Publishing Award, Faculty of Medicine and Dentistry, Palacký University Olomouc

1. Commentary

This part of my work represents our strong clinical intention to find new treatment options for our patients suffering from Pheochromocytoma/Paraganglioma. Our search was focused on treatment options novel for PHEO/PGL patients but already available for clinical use. We found a clinical trial organized at NIH Bethesda - "Indenoisoquinoline LMP400 for Advanced Solid Tumors and Lymphomas", clinical trials identifier - NCT01794104. We contacted the organizing committee and with their help - special thanks to Dr. Kummar and support of Division of Cancer Treatment and Diagnosis we were able to perform the following study. We thoroughly tested the LMP-400 in vitro and in vivo, utilizing many approaches, initial studies were focused on in vitro cytotoxicity of the drug as a condition for further testing, we were very happy about the results and satisfied with a low IC50 concentration of LMP-400 on PHEO/PGL animal cell models and two independent primary human cancer cell cultures derived from two sporadic PHEOs. After finishing the in vitro part all the forces went into the animal study, using the model previously described in our laboratory utilizing a spontaneously metastatic model of PHEO with bioluminescent imaging.

The only available curative treatment for PHEO/PGL is surgery. When the tumor is unresectable or metastases are present, systemic chemotherapy or radiopharmaceutical therapies are used. These treatment methods are aimed at stopping metastatic spread and decreasing tumor- and hormone-related events (e.g., spinal instability, cardiovascular complications, etc.) in order to improve quality of life and survival. A recent clinical review summarized the current and future therapeutic approaches for PHEO and PGL, dividing them into anti-proliferative therapeutic strategies and pro-apoptotic strategies. As recently suggested, targeting topoisomerase I (Top1) may represent an interesting "pro-apoptotic" option.

Topoisomerases are ubiquitous enzymes essential for replication and transcription. They control DNA supercoiling and entanglement, which makes them attractive targets for anticancer and antibacterial treatment.

LMP-400/Indotecan, an HCl salt of NSC 724998 developed by the National Cancer Institute (NCI), is currently undergoing clinical evaluation and represents one of the third generation Top1 inhibitors. Indenoisoquinolines were developed to overcome certain

limitations of camptothecin derivatives, which are the only group of Top1 inhibitors approved by the U.S. Food and Drug Administration (FDA) for the treatment of solid tumors (Topotecan, Irinotecan).

We conclude that LMP-400 is a promising treatment option for patients with metastatic PHEO and represents a potential candidate for future clinical trials involving patients with these tumors.

2. Introduction

According to the World Health Organization (WHO) tumor classification, pheochromocytomas (PHEOs) are tumors of neuroendocrine origin found in the adrenal glands. Closely related extra-adrenal tumors found along the sympathetic or parasympathetic chain are referred to as paragangliomas (PGLs) (1). At least 35% of these tumors are of familial origin, caused by pathogenic mutations in several genes. Recently discovered mutations in the gene for hypoxia inducible factor 2 alpha (HIF2α) associated with multiple PHEOs/PGLs (2-5) opened another line of thinking about their future treatment options and how hereditary tumors could be linked to the HIF-signaling pathway (6,7).

The only available curative treatment for PHEO/PGL is surgery. When the tumor is unresectable or metastases are present, systemic chemotherapy or radiopharmaceutical therapies are used (8-11). These treatment methods are aimed at stopping metastatic spread and decreasing tumor- and hormone-related events (e.g., spinal instability, cardiovascular complications, etc.) in order to improve quality of life and survival (12-14). The use of 131I-metaiodobenzylguanidine (131I-MIBG) might help stabilize disease and lower tumor burden; however, 131I-MIBG can only be used when the tumor shows uptake (15). Many traditional chemotherapeutic agents and regimens are used for the treatment of metastatic PHEO, with the combination of cyclophosphamide, vincristine, and dacarbazine (CVD) being the most commonly used of these regimens. Patients receiving CVD often show initial benefit, but the disease usually recurs/progresses, leading to an overall poor prognosis (12,16-19). A recent clinical review summarized the current and future therapeutic approaches for PHEO and PGL, dividing them into anti-proliferative therapeutic strategies and pro-apoptotic strategies (12,18,20). As recently suggested, targeting topoisomerase I

(Top1) may represent an interesting “pro-apoptotic” option (21).

Topoisomerases are ubiquitous enzymes essential for replication and transcription. They control DNA supercoiling and entanglement, which makes them attractive targets for anticancer and antibacterial treatment (22). Top1 inhibitors act as interfacial inhibitors by blocking Top1 functions, leading to DNA damage through the formation of double-strand breaks, which, if not repaired, lead to cell death (23,24). The presence of Top1 is necessary for camptothecin and non-camptothecin Top1 inhibitors (e.g., indenoisoquinolines) to exert their cytotoxic effects, as Top1 is their primary target (25). However, there have been other effects reported in connection to Top1 inhibition, namely an effect on the HIF-1 protein and HIF-1 transcription targets (26-30). The HIF proteins (HIFs) function as transcription factors, physiologically responding to changes in oxygen levels. In cancer biology, HIFs play crucial roles in several processes such as cancer cell migration, invasiveness, metastasis, and resistance to radio- and chemotherapy (31,32). The potential modulation of HIF-1α and HIF-2α expression might be of particular interest when treating PHEO/PGL, since the hypoxic/pseudohypoxic pathway has been widely studied in these tumors (31,33-36).

LMP-400/Indotecan, an HCl salt of NSC 724998 developed by the National Cancer Institute (NCI), is currently undergoing clinical evaluation and represents one of the third generation Top1 inhibitors (37). Indenoisoquinolines were developed to overcome certain limitations of camptothecin derivatives, which are the only group of Top1 inhibitors approved by the U.S. Food and Drug Administration (FDA) for the treatment of solid tumors (Topotecan, Irinotecan). The limitations of camptothecin derivatives include chemical instability, rapid diffusion from Top1-DNA cleavage complexes, and active export from cells by efflux pumps. LMP-400 overcomes these limitations (24,25,38). Use of camptothecin derivatives has been recently shown as a possible treatment option in an in vitro study by our collaborative group (21).

Two main pharmacodynamic targets can be evaluated in connection with indenoisoquinoline treatment: Top1 and H2A histone family, member X (γ-H2AX). The measurement of pretreatment levels of Top1 in tumor tissue could be a predictive marker for response to indenoisoquinoline treatment, and serve as a marker for patient selection. Correlation between Top1 levels and tumor response has been reported in previous studies (37,39-41). Furthermore, an observed decrease in Top1 levels upon treatment with Top1

inhibitors might serve as a biomarker of target engagement, as proposed by Pfister et al. (26,37,42). Another pharmacodynamic target that has been extensively validated in connection with indenoisoquinoline treatment is histone γ-H2AX (25,43). Phosphorylation of γ-H2AX occurs shortly after the formation of DNA double-strand breaks, and the signal strength correlates with the number of breaks formed. Its detection assay was developed and validated by the NCI for use in clinical trials using LMP-400 and other DNA-damaging agents (44).

Our knowledge of signaling pathways involved in PHEO/PGL has been broadened over the past few years, leading to the identification of several promising molecular targets (12,18,45). The results of single, targeted molecular therapies seem to be inconclusive, as reported in recent reviews (12,18). The lack of efficacy of certain agents may be due to compensatory signaling pathways (45). Combination approaches might overcome this issue, as well as decreasing the likelihood of development of drug resistance. In the present study, we report our initial experience with LMP-400 both in vitro and in vivo on established animal PHEO cell lines and primary cell cultures from human tumor tissue. Testing included, among others, studies of tumor cell growth inhibition, animal models, drug synergism, and modulation of two pharmacodynamic targets. Additionally, we analyzed the expression of HIF-1α in treated cells, since the HIF-1 is transcription factor important in PHEO/PGL tumor development. We conclude that LMP-400 is a promising treatment option for patients with metastatic PHEO and represents a potential candidate for future clinical trials involving patients with these tumors.

3. Material and Methods

Cell lines and reagents

Mouse PHEO cell lines (MPC, MTT, and MTT-Luc) were maintained in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, and antibiotics (Gibco-Life Technologies). A rat PHEO cell line (PC12) was maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (Gibco-Life Technologies). Cells were grown until 80% confluence and then detached using 0.05% trypsin/EDTA, resuspended, and counted to obtain the desired number.

Cells were grown in an incubator in a humidified atmosphere containing 5% CO2 at 37°C. For experiments in which cultivation under hypoxic conditions was necessary, the cells were cultured in a CO2/O2 incubator (MCO-5M; Panasonic), where the volumes of O2 and CO2 were 1% and 5%, respectively.

LMP-400 (Indotecan, NSC 743400) was provided by the Division of Cancer Treatment and Diagnosis, NCI (Rockville, Maryland, USA). Suberoylanilide hydroxamic acid (SAHA/vorinostat), cisplatin, and vincristine (vincristine sulfate) were purchased from Tocris Bioscience (R&D Systems, Inc.). All of the compounds were dissolved in dimethylsulfoxide (DMSO); stock solutions were stored at -20°C and thawed prior to use. Control samples were treated with culture medium.

Cell proliferation assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, referred to as the MTT assay. 15,000 cells per well were plated in 96-well plates and incubated for 24 hours before drug treatment. After 48 hours of drug treatment, MTT solution (1 mg/mL; Sigma Chemical Co.) was added and plates were incubated at 37°C for 3 hours before measuring the absorbance at 562 nm (Bio-TEK Instruments).

Human samples

Human PHEO/PGL tissue samples were obtained from patients who underwent surgery at our institution under the IRB-approved protocol 00-CH-0093 of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health (NIH), and all patients gave written informed consent. Normal human adrenal medullas were obtained from anonymous organ donors without evidence of adrenal tumors from the Department of Urology, School of Medicine, Comenius University, Bratislava, Slovakia.

Primary tumor cultures and tyrosine hydroxylase immunocytochemistry

The procedure was performed as previously described (46). Briefly, dissociated cells were plated at low density in RPMI medium with 15% fetal bovine serum and antibiotics. Cultures in control media or dosed with various concentrations of LMP-400 were maintained for 10 days, with the media replaced every other day. The cells were then fixed

and stained for tyrosine hydroxylase (TH). To measure drug-induced cytotoxicity, surviving TH-positive cells were counted.

Real-time PCR

MTT cells were grown to log phase (~80% confluence) before treatment with indicated concentrations of LMP-400 or control in both hypoxic and normoxic conditions for 8 and 24 hours. Control samples were treated with media. Real-time PCR was performed on a ViiA7 real-time PCR system (Applied Biosystems) according to the manufacturer's recommendation using the TaqMan™ detection system. TaqMan gene expression assays for Hif1α, Epas1, Hk2, Vegfa, Slc2a1α were purchased from Applied Biosystems. 18S rRNA by Applied Biosystems was used as an endogenous control. ^^CT values were plotted for the power.

Western Blotting

MTT cells were grown to log phase before treatment with indicated concentrations of LMP-400 for 8 hours in hypoxia or normoxia. Control samples were treated with media. Cells were washed twice with ice-cold PBS and lysed in a cell lysis buffer (Cell Signaling Technology) supplemented with complete protease inhibitor cocktail (Roche) and a Phosphatase Inhibitor Cocktail (Cell Signaling Technology). Protein concentrations were measured using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's recommendation. Proteins were separated by 4-20% gradient SDS-PAGE (Bio-Rad Laboratories) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Antibodies against HIF-1 (H-206, Santa Cruz Biotechnology, Inc.), phospho-Histone γ-H2AX (Millipore), β-Actin (Cell Signaling Technology), tyrosine hydroxylase (Immunostar), and topoisomerase I (BD Biosciences) were used. Proteins were visualized using the SuperSignal® West Femto Maximum Sensitivity Substrate and SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). Blots were analyzed using ImageJ 1.37v (Wayne Rasband, NIH).

Synergism analysis

Drug synergism was determined from median effect analysis equations developed by Chou-Talalay (47). Cell proliferation data were analyzed using CalcuSyn software (Biosoft,

UK). The Combination Index (CI) indicates additivity when CI = 0.8-1.2; synergism when CI < 0.8; and antagonism when CI > 1.2. The Dose Reduction Index (DRI) shows potential dose reduction of each single drug in synergistic combination at a given effect level achieved by combining these drugs (47).

Animal experiments and bioluminescence imaging

All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and approved by the NIH Animal Care and Use Committee (ACUC) (Animal Study Proposal #12–028 and PHS Assurance #A4149–01).

We utilized a spontaneously metastatic model of PHEO after subcutaneous (S.C.) injection of MTT cells constitutively expressing luciferase (MTT-Luc) in the right flank of female athymic nude mice (Taconic), as described previously (48). All bioluminescent data were collected and analyzed with a Xenogen IVIS system. The experiments were performed in the NIH Mouse Imaging Facility in accordance with ACUC regulations. In the initial study, we administered LMP-400 or placebo (vehicle) once a day for 5 consecutive days with a dose of 12mg/kg, starting 7 days after cell injection. In the following study, we administered 20 mg/kg once a week, starting 7 days after cell injection. The LMP-400 dilution, administration, and manipulation have been previously described (44).

Shortly after being received from vendor, mice in both (treated and control) groups developed an infection from Corynebacterium bovis and were equally treated with TMS (trimethoprim and sulfamethoxazole), followed by ampicillin in water.

Statistics

All in-vitro experiments were repeated at least 2 times. Following the ANOVA analyses, post-test pairwise comparisons were computed using either Student-Newman-Keuls posttest (for all pairwise comparisons) or Dunnett's test (comparing multiple treatments vs. a single control), using Prism 6 software (GraphPad Software Inc.). Time-course analyses of the animal experiements with bioluminescence imaging data were analyzed using mixed models on the log values, to handle the repeating measurements over time, using Stata: Release 12 software (StataCorp). These plots show 95% confidence intervals (CIs) rather than SEMs. To analyze the caliper-measurements of tumor size in

these same experiments, at specific weeks, the treated and control group values were compared using the 2-sample t-test on arcsinh-transformed values (necessary due to the substantial skewness of the values and the presence of 0 values). The data were plotted with SEM and considered significant when $P < 0.05$ (marked as *).

4. Results

LMP-400 inhibits the proliferation of mouse and rat PHEO cell lines

Initially, we evaluated the effect of LMP-400 on available PHEO cell lines and found that it inhibited the growth of established animal cell lines from mice (MPC and MTT cells) and rats (PC12 cells) in a dose-dependent manner (Figure 1). The MTT assay was additionally repeated for MPC and MTT cell lines in hypoxic conditions with minor changes in the IC50; in MPC cells, the change was from 0.025 μM in normoxia to 0.033 μM in hypoxia, while in MTT cells, IC50 changed from 0.04 μM to 0.094 μM (Supplementary Figure 1). We also tested the growth inhibition effect of LMP-400 given for various time intervals. After an 8-hour treatment of MTT cells with LMP-400, we did not observe an effect of the drug on more than 30% of cells at any concentration for the evaluated range (1, 0.1, 0.01 μM). After 24 hours of LMP-400 treatment, the 1 μM concentration inhibited the growth of ~41% cells; for 0.1 μM and 0.01 μM concentrations, the cell inhibition remained below 30%. The IC50 for MTT cells after 24 hours of treatment was 0.58 μM (Supplementary Figure 2).

Top1 levels in PHEO/PGL

First, we assessed the tumor content of the Top1 protein since it is known that Top1 protein is a criterion used successfully for the clinical application of LMP-400 (37). Thus, we evaluated the levels of Top1 in sporadic and genetically linked PHEOs/PGLs and compared them to levels of Top1 in normal human adrenal medulla. This analysis showed higher levels of Top1 in all the evaluated tumors compared to the adrenal medulla (Figure 3, Panel A). Similar levels of Top1 expression were observed in sporadic and von Hippel-Lindau (VHL)-mutated tumors; the highest levels of Top1 expression were found in samples from succinate dehydrogenase subunit B (SDHB)-mutated tumors. These tumors are known to have a high metastatic potential and worse outcome than other known familial

PHEOs/PGLs (2,49).

LMP-400 inhibits proliferation of primary PHEO cells

Based on the findings that human PHEOs/PGLs exhibit high levels of Top1, we initiated treatment with LMP-400 for ten consecutive days using two independent primary human cancer cell cultures derived from two sporadic PHEOs. Immunostaining for tyrosine hydroxylase (TH), an enzyme necessary for catecholamine production, was used to distinguish chromaffin cells from other cells in the primary cell culture. Figure 2, shows a concentration-dependent decrease in cell proliferation for cells treated with LMP-400.

LMP-400 decreases Top1 and increases γ-H2AX levels in MTT cells

Pharmacodynamic assays for Top1 and γ-H2AX were previously developed for clinical trials with LMP-400 (37,44). Thus, we evaluated the effect of LMP-400 on these potential biomarkers in MTT cells, measuring target proteins after 8 hours of treatment with several drug concentrations in normoxic and hypoxic conditions. The levels of Top1 in MTT cells decreased in a concentration-dependent fashion in both hypoxic and normoxic conditions. On the other hand, the levels of γ-H2AX, which were almost not present in control cells, peaked upon treatment with LMP-400. MTT cells showed stable levels of the HIF1α protein in both hypoxic and normoxic conditions. This expression was decreased after 8 hours of treatment with LMP-400 (Figure 3, Panel B).

LMP-400 affected expression of HIF-1 targets in MTT cells

To determine the effect of LMP-400 treatment on Hif1α gene expression in MTT cells, we treated these cells with increasing LMP-400 concentrations. To limit the potential effect of cell apoptosis on mRNA expression, we used concentrations that did not affect cell proliferation in more than 30% of the cells. mRNA was extracted after 8 and 24 hours of LMP-400 treatment in both normoxic and hypoxic conditions (Supplementary Figure 2).

After an 8-hour treatment, we did not observe a significant decrease in Hif1α expression, but a decrease in Hif1α expression was apparent after 24 hours of treatment in both hypoxia and normoxia (Supplementary Figure 3). Since HIFs serve as transcription factors, determining the significance of changes in their expression levels is best measured by evaluation of the expression levels of their target genes. We found a significant decrease in two very well-established preferential HIF1 targets at the highest concentration tested,

irrespective of time or oxygen conditions: solute carrier family 2, facilitated glucose transporter member 1 (Slc2a1), better known as glucose transporter type 1 (Glut1), and hexokinase 2 (Hk2) (Figure 4 and Supplementary Figure 4).

LMP-400 reduced tumor growth and metastatic potential in vivo

To elucidate the effects of LMP-400 in an in vivo model, we used a well-established model of spontaneously metastatic PHEO, taking advantage of MTT-Luc bioluminescence imaging (48). Seven days after subcutaneous implantation of MTT-Luc cells (day 1), we started 5 continuous days of 12 mg/kg LMP-400 intravenous application, which led to an overall significant decrease in tumor growth ($P = 0.0005$) when measured by bioluminescence. The subcutaneous injection of MTT-Luc cells allowed us to measure tumor growth externally by caliper, and this measurement also confirmed a significant effect of LMP-400 on growth in vivo ($P = 0.003$ for week 3; $P = 0.015$ for week 4). Over time, the implanted cells started to migrate and metastases developed. After the mice had been euthanized, the lungs and liver were harvested and bioluminescence measurements were performed, showing that LMP-400 decreased the development of metastases (in lungs $P = 0.002$; in liver $P = 0.091$). A consecutive study with an alternative dosing schedule of 20 mg/kg once a week also showed a significant difference in tumor growth ($P = 0.044$, bioluminescence measurement) when compared to a group that received only a vehicle. The significance of the bioluminescence measurement was confirmed by external caliper measurements (week 5 $P = 0.037$) (Figure 5).

LMP-400 as a part of combination treatment

CVD treatment represents one of the best available chemotherapeutic regimens for patients with metastatic PHEO/PGL, although it can be modified, as mentioned earlier. Because this combination cannot be tested in vitro (e.g. Dacarbazine, which is an essential part of this combination is a pro drug that needs to be activated by the liver), we attempted to closely simulate CVD treatment by combining cisplatin and vincristine. We aimed to show LMP-400's potential by adding it to this blend. Testing this combination in concentrations of original single drugs (CIS, VIN, LMP-400) below and above their IC50s showed high synergism at lower concentrations, which turned into an additive effect at the second-highest concentration. At the highest concentration, where the effect of a single drug alone was already very potent, the synergism was not present. Table 1 shows CI and DRI

values when the synergism was evaluated between LMP-400, vincristine, and cisplatin.

5. Discussion

In the present study, we evaluated the complex effect exerted by LMP-400 on PHEO/PGL in vivo and in vitro. LMP-400 distinctly inhibited the growth of human and animal PHEO/PGL cells. The effect of the agent on both the pharmacodynamic markers evaluated (Top1 and γ-H2AX) was found to be significant in MTT cells. The HIF1α protein and HIF1 transcriptional targets were also significantly affected by LMP-400 in MTT cells.

FDA approved Top1inhibitors, topotecan and irinotecan, are derivatives of camptothecins. Although they both target Top1, their clinical use is different. While topotecan is used to treat ovarian and lung cancers, irinotecan has been shown to be effective in the treatment of colon cancer (22). Pharmacodynamic and clinical limitations of camptothecin derivatives led to the development of non-camptothecin Top1 inhibitors, including LMP-400 (24). Top1 inhibitors were described as interfacial inhibitors that prevent Top1 functions, leading to double-strand DNA breaks, which, if not repaired, lead to cell death (24,50). It was proposed that pretreatment levels of Top1 might be an important factor in determining the effectiveness of Top1 inhibitors and thus predicting treatment response (37). Our evaluation of Top1 protein levels in several types of PHEO/PGL showed the highest levels of this protein (compared to normal adrenal medulla) in SDHB-mutated tumors. This is of interest since Top 1 could represent a therapeutic target in this disease and lead to the development of more effective drugs for this population.

First, we tested the efficacy of LMP-400 in vitro using MPC, MTT, and PC12 cell lines. MPC cells were derived from an Nf1 knock-out mouse that developed PHEO; the MTT cell line was derived from a liver metastasis of an MPC tumor and is thus considered to be the most aggressive available PHEO cell line, which guided our decision to use this cell line for most parts of this complex drug evaluation study. The PC12 cell line is of rat origin and is used as a model cell line for neuroendocrine tumors (51,52). LMP-400 showed efficacy in all animal PHEO cell lines, with IC50s in the tens of nanomolar concentration range. There is no available human cell line for PHEO/PGL, which would tremendously enhance the possibilities in the search for new therapeutic options. Despite extensive ongoing research, none of the attempts has been successful. This makes primary cell cultures prepared from

tumor tissue obtained from NIH patient surgeries the best option for current drug testing, although animal and human pheochromocytoma cells are known to have different growth rate. As we previously mentioned in the results section, LMP-400 substantially inhibited growth of these primary cell cultures.

These studies suggest that LMP-400 might be a promising therapeutic avenue for PHEO/PGL. Since in vivo studies are essential for introducing a new drug into clinical practice, we took advantage of a metastatic PHEO/PGL animal model that has been used in our previous studies and allows for non-invasive, repeatable, and reproducible in vivo tumor measurement (48). In the initial animal study, when the drug was applied constitutively for 5 days, a statistically significant effect was reached, established by the measurement of bioluminescence as well as external tumor measurement by caliper. LMP-400 also showed an effect on the development of metastases. The same 5-day dosing schedule was previously tested in a model study of LMP-400 using mice bearing A375 tumor xenografts (human malignant melanoma) (44). An alternative for this monthly dosing plan is a weekly schedule using a higher single dose of 20 mg/kg. We in fact implemented this scheme of dosing into our study, and tumor growth inhibition was also significant. However, there was an obvious difference between the two approaches. Monthly dosing, with the cumulative dose applied in the early stage of MTT-Luc cell tumor development, led to very significant efficacy at the beginning of the study, which went then down gradually. In contrast, weekly dosing required a longer time to exert a significant effect, resulting in decreased formation of MTT-Luc cell tumors. We believe that the observed difference between the two dosing schemes can be caused by the aggressiveness of MTT cells.

The use of combined chemotherapeutic approaches can be beneficial for patients if the drugs show a synergistic effect, eventually leading to a reduction in drug dose with the mitigation of some side effects and reduction in the development of resistance, in contrast to full dosing of the drug (47). Despite its weaknesses, CVD chemotherapy has been found to be the best available chemotherapy regimen for PHEO/PGL (12,16-18). We attempted to imitate the CVD regimen in in vitro conditions using vincristine and cisplatin with the addition of LMP-400 to this combination. This treatment led to decreased cell growth in concentrations below and above the respective IC50s, suggesting the possibility of adding LMP-400 to the CVD regimen. We think that the synergism at low doses might be potentially important.

For use in clinical trials with LMP-400, two assays focused on the pharmacodynamic markers Top1 and γ-H2AX were developed (37,44). We tested these markers when treating MTT cells with LMP-400 and observed a dramatic increase in γ-H2AX, which indicates the development of DNA damage after the treatment and can be considered as induction of early chromatin modification following initiation of DNA fragmentation during apoptosis (53). We also observed a decrease in Top1. Both of these effects were achieved in both normoxic and hypoxic conditions. Showing the LMP-400 effectiveness in hypoxic conditions is of great importance, since hypoxic conditions are associated with tumor aggressiveness, progression, and acquired resistance to treatment (54).

It was previously shown that topoisomerase inhibitors also deliver effects beyond cytotoxicity. When LMP-400 was tested at lower concentrations (inhibiting the growth of less than 30% of cells in a given time period), a decrease in HIF1α protein was observed. HIF-1/2α were proposed to be the mediators of hypoxic signaling in VHL- and SDHx-mutated PHEOs/PGLs (3,55). In an unsupervised analysis of the transcriptional profile of these tumors, reduced oxidoreductase and angiogenesis/hypoxia were seen, suggesting that these tumors have similar profiles, leading to their categorization as Cluster 1 tumors. Activation of receptor tyrosine kinases, possibly leading to increased transcription of HIF1α is, however, more common for Cluster 2, consisting of PHEO/PGL with germline mutations in several other susceptibility genes (mainly RET, NF1, and MAX). Sporadic PHEOs/PGLs are equally distributed in both clusters (34,56). Thus, targeting HIF-1α might be of potential interest for all PHEOs/PGLs (36,45). Though levels of HIF-1α and HIF-2α in PHEO/PGL, once referred to as “rivaling siblings” (32), were previously evaluated, no unifying pattern was found (33,57,58). Therefore, the balance between these two proteins in PHEO/PGL is still inconclusive, but may possibly also depend on the development stage of the tumor. Changes in HIF-1 are not specific to Top1 inhibitors, since similar results were obtained after treatment with Top2 inhibitors, but specific genes are likely to respond individually to topoisomerase inhibition. The response can result directly from enzyme inhibition or might be due to a secondary mechanism (59). Previous reports showed that HIF-1 changes are not transcriptional, which is not consistent with the present study, since we found changes in the mRNA levels of HIF1 targets after prolonged treatment. Nevertheless, the specific pathway causing changes in HIF-1 levels after topoisomerase treatment needs to be further studied. The presence of Top1 does seem to be a unifying condition for its exertion (27,29,30). We have also evaluated the effects of 1 μM LMP-400 on HIF-1 target gene

expression. Since this concentration is already toxic after prolonged treatment, we treated the cells with the drug for only 8 and 24 hours. The data show a consistent expressional decrease in the known HIF-1 target genes including Glut1, Hk2, and Vegfa under hypoxic conditions after both 8 and 24 hours (Figure 4; Supplemental Figures 4 and 5). In normoxia, mRNA levels of all target genes were also significantly lower in all conditions when compared to control cells, with the exception of Vegfa after an 8-hour treatment. Expression of Hif2α was increased after an 8-hour treatment, irrespective of oxygen conditions. This increase was not present after prolonged treatment (Supplemental Figure 7). Due to this observation, we were eager to evaluate its transcriptional targets, looking for a possible interplay between HIFs. Despite the fact that we evaluated Epo mRNA after 8 and 24 hours of hypoxia, we did not observe any change in its expression (Supplemental Figure 8). It has been shown that Top1 inhibitors, rather than decreasing the expression of several genes, increase the mRNA levels of prostaglandin-endoperoxide synthase 2, also known as cyclooxygene 2 (Ptgs2, Cox-2 respectively). This was discussed in connection with potential NF-κB activation (30). We also evaluated this gene but only found a significant increase in its mRNA levels under normoxic conditions after treatment with 1 μM of LMP-400 for 8 hours (Supplemental Figure 6).

In conclusion, LMP-400, whose effects were thoroughly evaluated on the best available PHEO/PGL models, represents a promising step in the search for new treatment options for PHEO/PGL patients.

6. References: Experimental Part (Authors listed in *Endocrinology* order)

1. DeLellis RA. Pathology and genetics of tumours of endocrine organs. Lyon: IARC Press.

2. Karasek D, Shah U, Frysak Z, Stratakis C, Pacak K. An update on the genetics of pheochromocytoma. J Hum Hypertens 2013; 27:141-147

3. Pacak K, Jochmanova I, Prodanov T, Yang C, Merino MJ, Fojo T, Prchal JT, Tischler AS, Lechan RM, Zhuang Z. New syndrome of paraganglioma and somatostatinoma associated with polycythemia. J Clin Oncol 2013; 31:1690-1698

4. Toledo RA, Qin Y, Srikantan S, Morales NP, Li Q, Deng Y, Kim SW, Pereira MA, Toledo SP, Su X, Aguiar RC, Dahia PL. In vivo and in vitro oncogenic effects of HIF2A mutations in pheochromocytomas and paragangliomas. Endocrine-related cancer 2013; 20:349-359

5. Comino-Mendez I, de Cubas AA, Bernal C, Alvarez-Escola C, Sanchez-Malo C, Ramirez-Tortosa CL, Pedrinaci S, Rapizzi E, Ercolino T, Bernini G, Bacca A, Leton R, Pita G, Alonso MR, Leandro-Garcia LJ, Gomez-Grana A, Inglada-Perez L, Mancikova V, Rodriguez-Antona C, Mannelli M, Robledo M, Cascon A. Tumoral EPAS1 (HIF2A) mutations explain sporadic pheochromocytoma and paraganglioma in the absence of erythrocytosis. Hum Mol Genet 2013; 22:2169-2176

6. Jochmanova I, Lazurova I. A new twist in neuroendocrine tumor research: Pacak-Zhuang syndrome, HIF-2alpha as the major player in its pathogenesis and future therapeutic options. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia 2014; 158:175-180

7. Vicha A, Musil Z, Pacak K. Genetics of pheochromocytoma and paraganglioma syndromes: new advances and future treatment options. Current opinion in endocrinology, diabetes, and obesity 2013; 20:186-191

8. Adjalle R, Plouin PF, Pacak K, Lehnert H. Treatment of malignant pheochromocytoma. Horm Metab Res 2009; 41:687-696

9. Ayala-Ramirez M, Feng L, Habra MA, Rich T, Dickson PV, Perrier N, Phan A, Waguespack S, Patel S, Jimenez C. Clinical benefits of systemic chemotherapy for patients with metastatic pheochromocytomas or sympathetic extra-adrenal

paragangliomas: insights from the largest single-institutional experience. Cancer 2012; 118:2804-2812

10. Baudin E, Habra MA, Deschamps F, Cote G, Dumont F, Cabanillas M, Arfi-Roufe J, Berdelou A, Moon B, Al Ghuzlan A, Patel S, Leboulleux S, Jimenez C. THERAPY OF ENDOCRINE DISEASE: Treatment of malignant pheochromocytoma and paraganglioma. European journal of endocrinology / European Federation of Endocrine Societies 2014; 171:R111-R122

11. Taieb D, Kaliski A, Boedeker CC, Martucci V, Fojo T, Adler JR, Jr., Pacak K. Current approaches and recent developments in the management of head and neck paragangliomas. Endocrine reviews 2014:er20141026

12. Plouin PF, Fitzgerald P, Rich T, Ayala-Ramirez M, Perrier ND, Baudin E, Jimenez C. Metastatic pheochromocytoma and paraganglioma: focus on therapeutics. Horm Metab Res 2012; 44:390-399

13. Taieb D, Timmers HJ, Hindie E, Guillet BA, Neumann HP, Walz MK, Opocher G, de Herder WW, Boedeker CC, de Krijger RR, Chiti A, Al-Nahhas A, Pacak K, Rubello D. EANM 2012 guidelines for radionuclide imaging of phaeochromocytoma and paraganglioma. European journal of nuclear medicine and molecular imaging 2012; 39:1977-1995

14. Timmers HJ, Chen CC, Carrasquillo JA, Whatley M, Ling A, Havekes B, Eisenhofer G, Martiniova L, Adams KT, Pacak K. Comparison of 18F-fluoro-L-DOPA, 18F-fluoro-deoxyglucose, and 18F-fluorodopamine PET and 123I-MIBG scintigraphy in the localization of pheochromocytoma and paraganglioma. The Journal of clinical endocrinology and metabolism 2009; 94:4757-4767

15. Ilias I, Divgi C, Pacak K. Current role of metaiodobenzylguanidine in the diagnosis of pheochromocytoma and medullary thyroid cancer. Semin Nucl Med 2011; 41:364-368

16. Huang H, Abraham J, Hung E, Averbuch S, Merino M, Steinberg SM, Pacak K, Fojo T. Treatment of malignant pheochromocytoma/paraganglioma with cyclophosphamide, vincristine, and dacarbazine: recommendation from a 22-year follow-up of 18 patients. Cancer 2008; 113:2020-2028

17. Scholz T, Eisenhofer G, Pacak K, Dralle H, Lehnert H. Clinical review: Current treatment of malignant pheochromocytoma. The Journal of clinical endocrinology and metabolism 2007; 92:1217-1225

18. Matro J, Giubellino A, Pacak K. Current and Future Therapeutic Approaches for Metastatic Pheochromocytoma and Paraganglioma: Focus on SDHB Tumors. Horm Metab Res 2013; 45:147-153

19. Jimenez C, Rohren E, Habra MA, Rich T, Jimenez P, Ayala-Ramirez M, Baudin E. Current and Future Treatments for Malignant Pheochromocytoma and Sympathetic Paraganglioma. Curr Oncol Rep 2013;

20. Jimenez C, Rohren E, Habra MA, Rich T, Jimenez P, Ayala-Ramirez M, Baudin E. Current and future treatments for malignant pheochromocytoma and sympathetic paraganglioma. Curr Oncol Rep 2013; 15:356-371

21. Powers JF, Korgaonkar PG, Fliedner S, Giubellino A, Pacak K, Sahagian GG, Tischler AS. Cytocidal activities of topoisomerase 1 inhibitors and 5-azacytidine against pheochromocytoma/paraganglioma cells in primary human tumor cultures and mouse cell lines. PLoS One 2014; 9:e87807

22. Pommier Y. Drugging topoisomerases: lessons and challenges. ACS Chem Biol 2013; 8:82-95

23. Sordet O, Khan QA, Kohn KW, Pommier Y. Apoptosis induced by topoisomerase inhibitors. Current medicinal chemistry Anti-cancer agents 2003; 3:271-290

24. Pommier Y, Cushman M. The indenoisoquinoline noncamptothecin topoisomerase I inhibitors: update and perspectives. Mol Cancer Ther 2009; 8:1008-1014

25. Antony S, Agama KK, Miao ZH, Takagi K, Wright MH, Robles AI, Varticovski L, Nagarajan M, Morrell A, Cushman M, Pommier Y. Novel indenoisoquinolines NSC 725776 and NSC 724998 produce persistent topoisomerase I cleavage complexes and overcome multidrug resistance. Cancer Res 2007; 67:10397-10405

26. Beidler DR, Cheng YC. Camptothecin induction of a time- and concentration-dependent decrease of topoisomerase I and its implication in camptothecin activity. Mol Pharmacol 1995; 47:907-914

27. Choi YJ, Rho JK, Lee SJ, Jang WS, Lee SS, Kim CH, Lee JC. HIF-1alpha modulation by topoisomerase inhibitors in non-small cell lung cancer cell lines. J Cancer Res Clin Oncol 2009; 135:1047-1053

28. Guerin E, Raffelsberger W, Pencreach E, Maier A, Neuville A, Schneider A, Bachellier P, Rohr S, Petitprez A, Poch O, Moras D, Oudet P, Larsen AK, Gaub MP, Guenot D. In vivo topoisomerase I inhibition attenuates the expression of hypoxia-inducible factor 1alpha target genes and decreases tumor angiogenesis. Mol Med 2012; 18:83-94

29. Rapisarda A, Uranchimeg B, Sordet O, Pommier Y, Shoemaker RH, Melillo G. Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: mechanism and therapeutic implications. Cancer Res 2004; 64:1475-1482

30. Rapisarda A, Uranchimeg B, Scudiero DA, Selby M, Sausville EA, Shoemaker RH, Melillo G. Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 2002; 62:4316-4324

31. Semenza GL. Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. Trends Pharmacol Sci 2012; 33:207-214

32. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer 2012; 12:9-22

33. Pollard PJ, El-Bahrawy M, Poulsom R, Elia G, Killick P, Kelly G, Hunt T, Jeffery R, Seedhar P, Barwell J, Latif F, Gleeson MJ, Hodgson SV, Stamp GW, Tomlinson IP, Maher ER. Expression of HIF-1alpha, HIF-2alpha (EPAS1), and their target genes in paraganglioma and pheochromocytoma with VHL and SDH mutations. The Journal of clinical endocrinology and metabolism 2006; 91:4593-4598

34. Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, Barontini M, Kung AL, Sanso G, Powers JF, Tischler AS, Hodin R, Heitritter S, Moore F, Dluhy R, Sosa JA, Ocal IT, Benn DE, Marsh DJ, Robinson BG, Schneider K, Garber J, Arum SM, Korbonits M, Grossman A, Pigny P, Toledo SP, Nose V, Li C, Stiles CD. A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. PLoS Genet 2005; 1:72-80

35. Lopez-Jimenez E, Gomez-Lopez G, Leandro-Garcia LJ, Munoz I, Schiavi F, Montero-Conde C, de Cubas AA, Ramires R, Landa I, Leskela S, Maliszewska A, Inglada-Perez L,

de la Vega L, Rodriguez-Antona C, Leton R, Bernal C, de Campos JM, Diez-Tascon C, Fraga MF, Boullosa C, Pisano DG, Opocher G, Robledo M, Cascon A. Research resource: Transcriptional profiling reveals different pseudohypoxic signatures in SDHB and VHL-related pheochromocytomas. Mol Endocrinol 2010; 24:2382-2391

36. Jochmanova I, Chunzhang Y, Zhuang Z, Pacak K. Hypoxia-Inducible Factor Signaling in Pheochromocytoma: Turning the Rudder in the Right Direction. Journal of the National Cancer Institute 2013;

37. Pfister TD, Hollingshead M, Kinders RJ, Zhang Y, Evrard YA, Ji J, Khin SA, Borgel S, Stotler H, Carter J, Divelbiss R, Kummar S, Pommier Y, Parchment RE, Tomaszewski JE, Doroshow JH. Development and validation of an immunoassay for quantification of topoisomerase I in solid tumor tissues. PLoS One 2012; 7:e50494

38. Cinelli MA, Reddy PV, Lv PC, Liang JH, Chen L, Agama K, Pommier Y, van Breemen RB, Cushman M. Identification, synthesis, and biological evaluation of metabolites of the experimental cancer treatment drugs indotecan (LMP400) and indimitecan (LMP776) and investigation of isomerically hydroxylated indenoisoquinoline analogues as topoisomerase I poisons. J Med Chem 2012; 55:10844-10862

39. Meisenberg C, Ward SE, Schmid P, El-Khamisy SF. TDP1/TOP1 Ratio as a Promising Indicator for the Response of Small Cell Lung Cancer to Topotecan. Journal of cancer science & therapy 2014; 6:258-267

40. Giovanella BC, Stehlin JS, Wall ME, Wani MC, Nicholas AW, Liu LF, Silber R, Potmesil M. DNA topoisomerase I--targeted chemotherapy of human colon cancer in xenografts. Science 1989; 246:1046-1048

41. Braun MS, Richman SD, Quirke P, Daly C, Adlard JW, Elliott F, Barrett JH, Selby P, Meade AM, Stephens RJ, Parmar MK, Seymour MT. Predictive biomarkers of chemotherapy efficacy in colorectal cancer: results from the UK MRC FOCUS trial. J Clin Oncol 2008; 26:2690-2698

42. Pfister TD, Reinhold WC, Agama K, Gupta S, Khin SA, Kinders RJ, Parchment RE, Tomaszewski JE, Doroshow JH, Pommier Y. Topoisomerase I levels in the NCI-60 cancer cell line panel determined by validated ELISA and microarray analysis and correlation with indenoisoquinoline sensitivity. Mol Cancer Ther 2009; 8:1878-1884

43. Pommier Y. DNA topoisomerase I inhibitors: chemistry, biology, and interfacial inhibition. Chem Rev 2009; 109:2894-2902

44. Kinders RJ, Hollingshead M, Lawrence S, Ji J, Tabb B, Bonner WM, Pommier Y, Rubinstein L, Evrard YA, Parchment RE, Tomaszewski J, Doroshow JH. Development of a validated immunofluorescence assay for gammaH2AX as a pharmacodynamic marker of topoisomerase I inhibitor activity. Clin Cancer Res 2010; 16:5447-5457

45. Nolting S, Grossman AB. Signaling pathways in pheochromocytomas and paragangliomas: prospects for future therapies. Endocr Pathol 2012; 23:21-33

46. Giubellino A, Bullova P, Nolting S, Turkova H, Powers JF, Liu Q, Guichard S, Tischler AS, Grossman AB, Pacak K. Combined inhibition of mTORC1 and mTORC2 signaling pathways is a promising therapeutic option in inhibiting pheochromocytoma tumor growth: in vitro and in vivo studies in female athymic nude mice. Endocrinology 2013; 154:646-655

47. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006; 58:621-681

48. Giubellino A, Woldemichael GM, Sourbier C, Lizak MJ, Powers JF, Tischler AS, Pacak K. Characterization of two mouse models of metastatic pheochromocytoma using bioluminescence imaging. Cancer Lett 2012; 316:46-52

49. Amar L, Baudin E, Burnichon N, Peyrard S, Silvera S, Bertherat J, Bertagna X, Schlumberger M, Jeunemaitre X, Gimenez-Roqueplo AP, Plouin PF. Succinate dehydrogenase B gene mutations predict survival in patients with malignant pheochromocytomas or paragangliomas. The Journal of clinical endocrinology and metabolism 2007; 92:3822-3828

50. Pommier Y, Marchand C. Interfacial inhibitors: targeting macromolecular complexes. Nat Rev Drug Discov 2012; 11:25-36

51. Korpershoek E, Pacak K, Martiniova L. Murine models and cell lines for the investigation of pheochromocytoma: applications for future therapies? Endocr Pathol 2012; 23:43-54

52. Martiniova L, Lai EW, Elkahloun AG, Abu-Asab M, Wickremasinghe A, Solis DC, Perera SM, Huynh TT, Lubensky IA, Tischler AS, Kvetnansky R, Alesci S, Morris JC,

Pacak K. Characterization of an animal model of aggressive metastatic pheochromocytoma linked to a specific gene signature. Clin Exp Metastasis 2009; 26:239-250

53. Rogakou EP, Nieves-Neira W, Boon C, Pommier Y, Bonner WM. Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. The Journal of biological chemistry 2000; 275:9390-9395

54. Tatum JL, Kelloff GJ, Gillies RJ, Arbeit JM, Brown JM, Chao KS, Chapman JD, Eckelman WC, Fyles AW, Giaccia AJ, Hill RP, Koch CJ, Krishna MC, Krohn KA, Lewis JS, Mason RP, Melillo G, Padhani AR, Powis G, Rajendran JG, Reba R, Robinson SP, Semenza GL, Swartz HM, Vaupel P, Yang D, Croft B, Hoffman J, Liu G, Stone H, Sullivan D. Hypoxia: importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. Int J Radiat Biol 2006; 82:699-757

55. Kaelin WG, Jr. Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer 2002; 2:673-682

56. Gimenez-Roqueplo AP, Dahia PL, Robledo M. An update on the genetics of paraganglioma, pheochromocytoma, and associated hereditary syndromes. Horm Metab Res 2012; 44:328-333

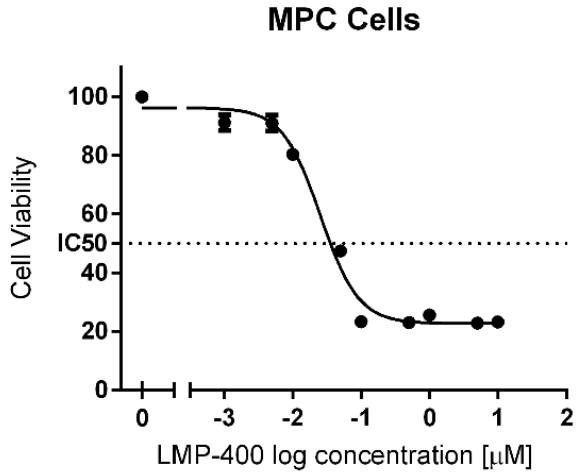
57. Eisenhofer G, Huynh TT, Pacak K, Brouwers FM, Walther MM, Linehan WM, Munson PJ, Mannelli M, Goldstein DS, Elkahloun AG. Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome. Endocrine-related cancer 2004; 11:897-911

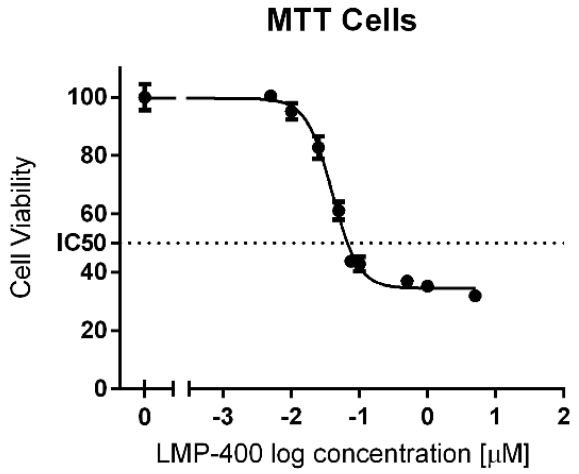
58. Favier J, Briere JJ, Burnichon N, Riviere J, Vescovo L, Benit P, Giscos-Douriez I, De Reynies A, Bertherat J, Badoual C, Tissier F, Amar L, Libe R, Plouin PF, Jeunemaitre X, Rustin P, Gimenez-Roqueplo AP. The Warburg effect is genetically determined in inherited pheochromocytomas. PLoS One 2009; 4:e7094

59. Collins I, Weber A, Levens D. Transcriptional consequences of topoisomerase inhibition. Mol Cell Biol 2001; 21:8437-8451

7. Figures and Tables

Figure 1. Tumor cell growth inhibition by LMP-400. Figure 1 shows tumor cell viability, measured by MTT assay, after a 48-hour treatment of established animal PHEO cell lines with LMP-400.





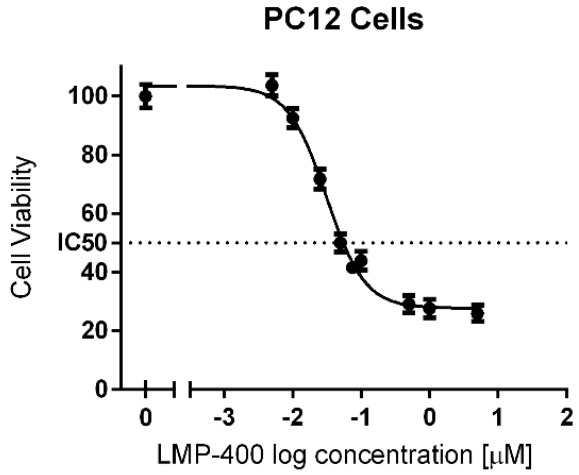


Figure 2. Primary cell culture growth inhibition by LMP-400. Figure 2. shows the effect of LMP-400 on a primary cell culture, with decreasing concentrations of the drug: a) control b) 1 μM c) 0.1 μM d 0.01 μM. The primary culture was derived from a 10 cm x 9 cm x 8 cm PHEO in a 53-year-old patient of Greek descent with typical clinical symptoms. Genetic testing for succinate dehydrogenase subunits B, C, and D was negative; other testing was not performed.

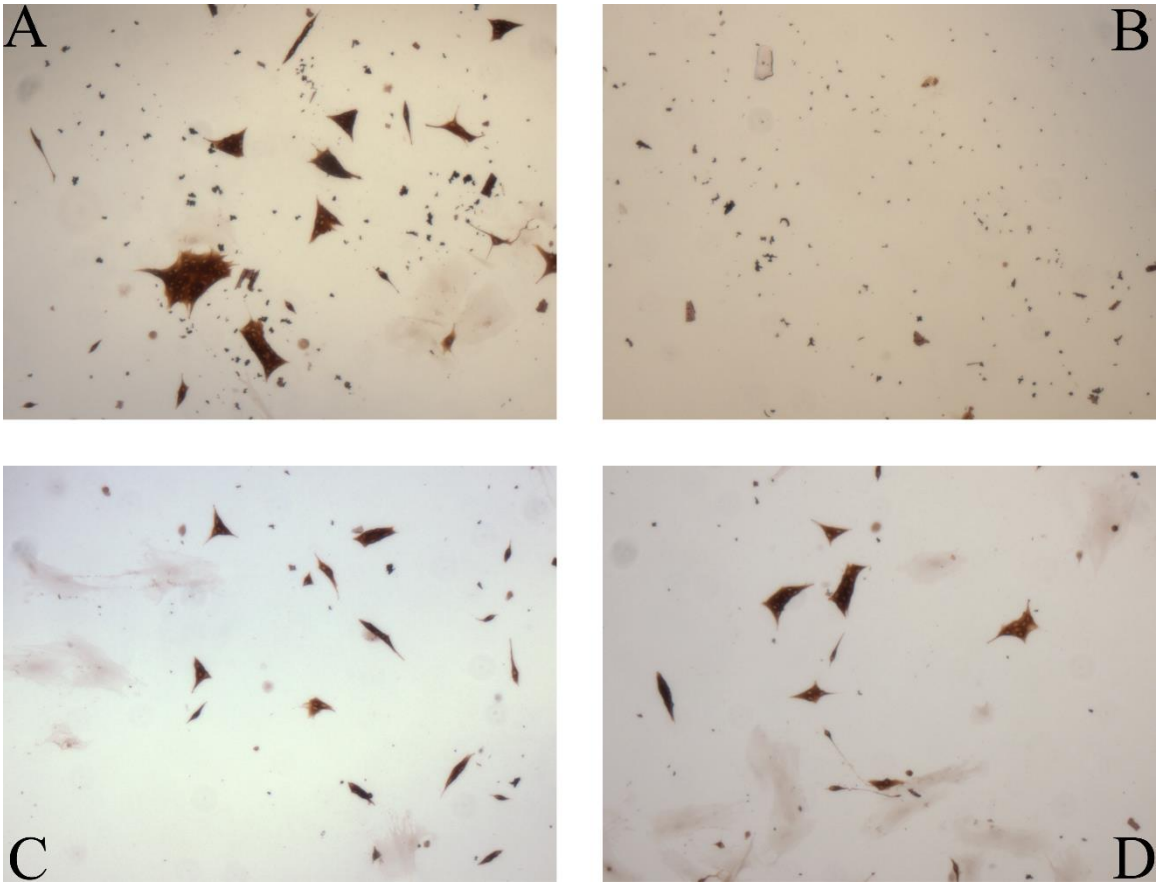


Figure 3. Protein analyses. Panel A shows variable amounts of the Top1 protein in tumors with different genotypes when compared to normal adrenal medulla (NAM). Panel B shows the levels of Top1, γ-H2AX, and HIF-1α evaluated after 8 hours of treatment with LMP-400 in hypoxic (1% O2, red curve) and normoxic conditions (21% O2, blue curve). Each oxygen condition has its own control, as presented on the figure.

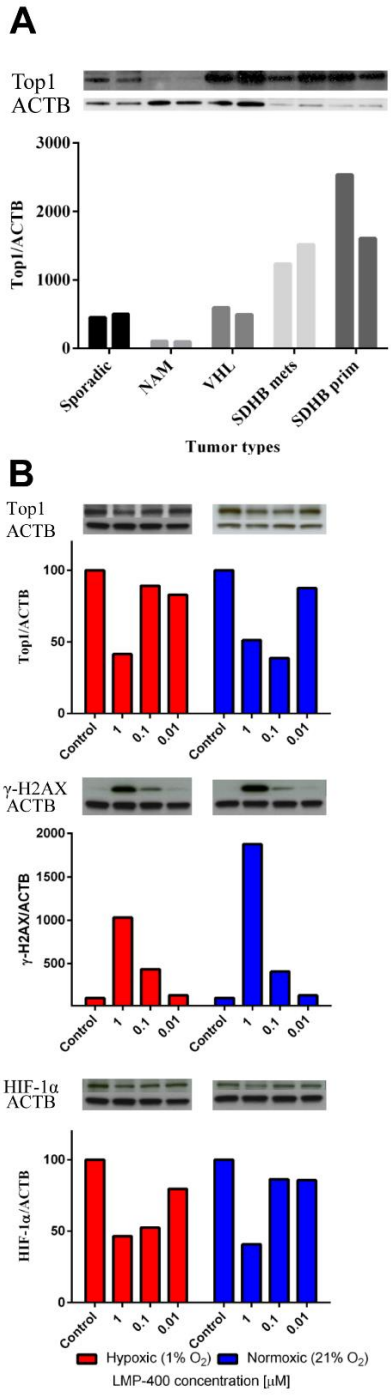


Figure 4. Glut1 (Slc2a1α) mRNA level changes. Figure 4 depicts changes in mRNA levels of Glut1 (Slc2a1α) upon 8- and 24-hour treatments with LMP-400 in hypoxic (1% O2, red column) and normoxic conditions (21% O2, blue column).

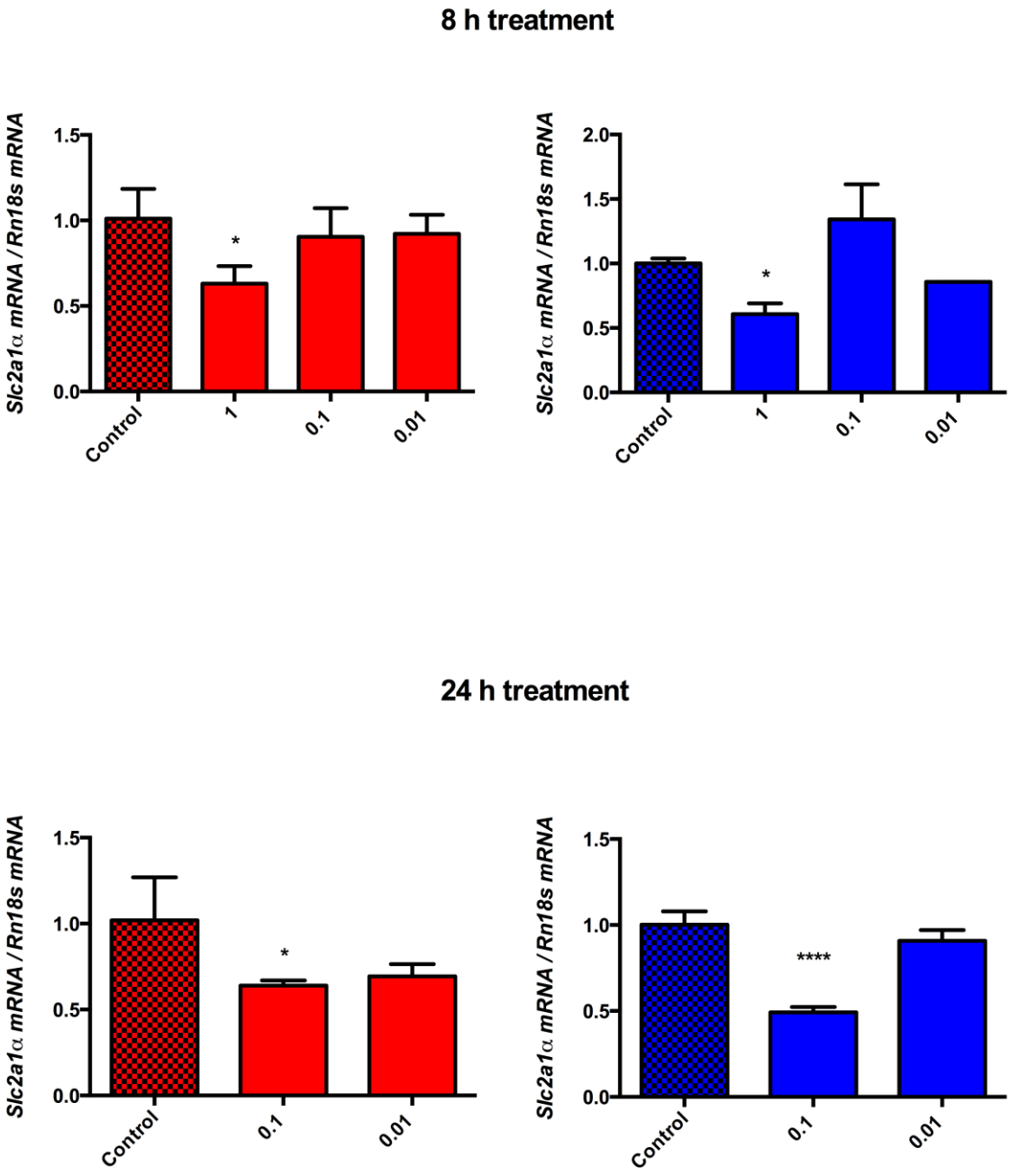


Figure 5. In vivo study of LMP-400. Figure 5 shows the effect of LMP-400 on tumor growth when dosed for 5 consecutive days (panel A) or once a week (panel B).

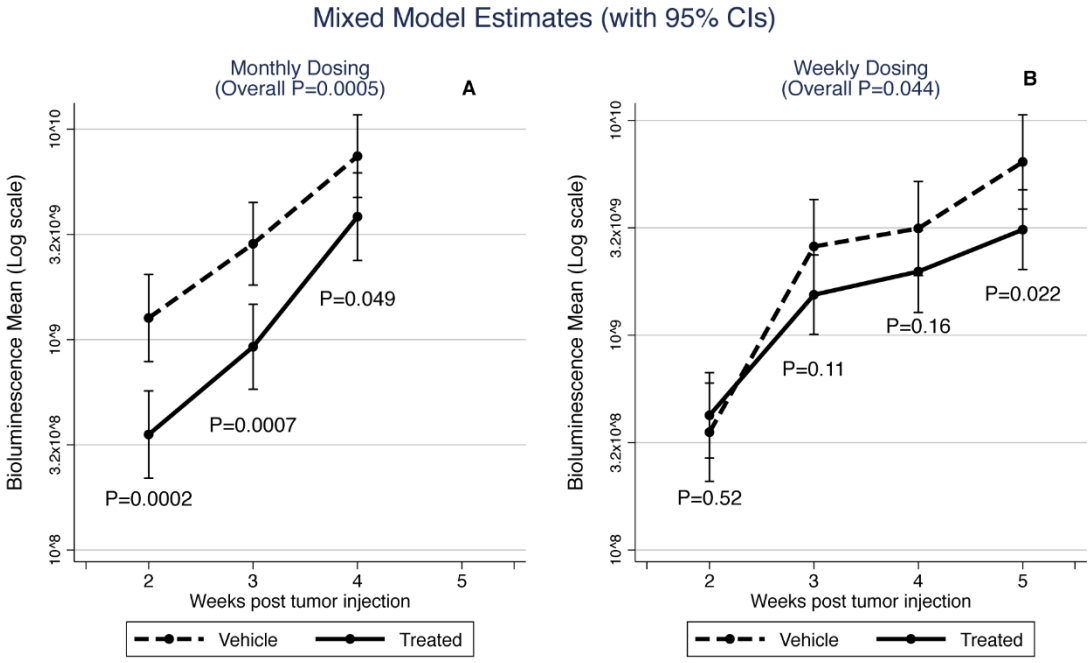
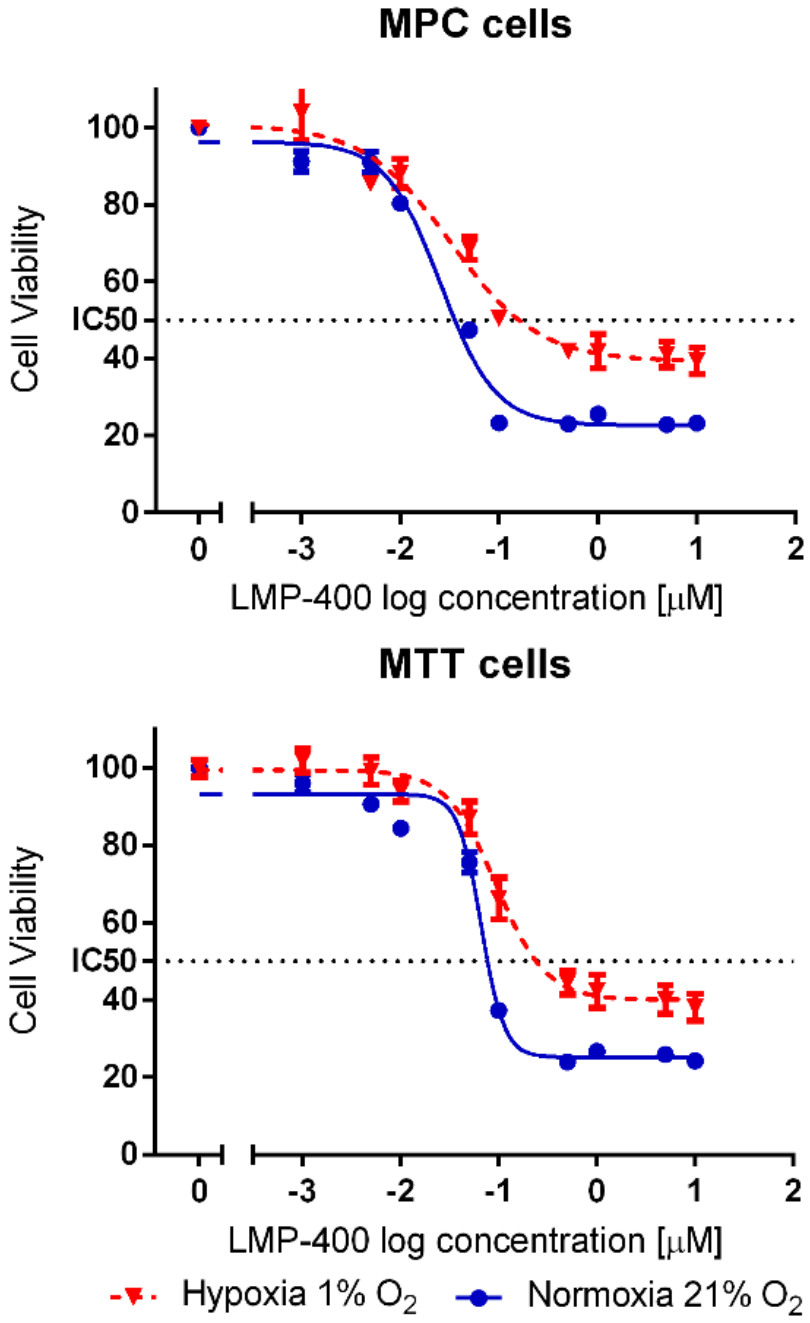


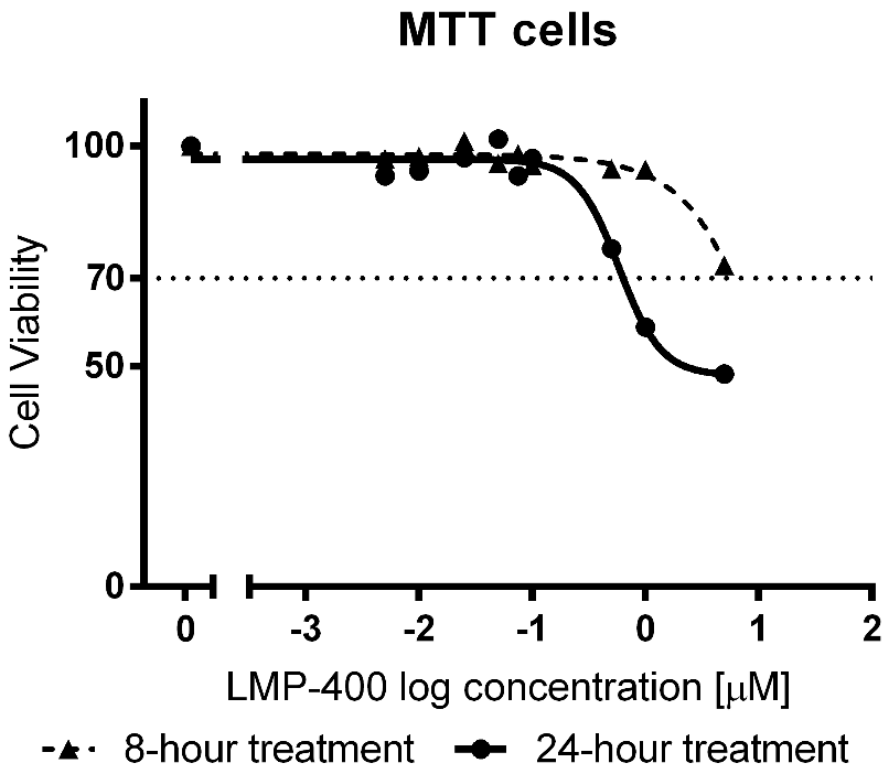
Table 1. Combinational testing. Table 1 shows the fractions of affected cells (Fa) with different doses of tested drugs on MTT cell survival after 48 hours of treatment with corresponding values of the Combinational Index (CI) and Dose Reduction Index (DRI).

Drug dose [μM]			Fa	CI	Dose Reduction Index (DRI)		
LMP-400	VIN	CIS			LMP-400	VIN	CIS
0.001	0.001	0.1	0.173937	0.653	9.752	17.008	2.034
0.005	0.005	0.5	0.345735	0.48	13.447	9.032	3.394
0.01	0.01	1	0.456195	0.395	17.732	7.376	4.925
0.05	0.05	5	0.777163	0.202	70.506	6.693	26.315
0.1	0.1	10	0.823222	0.274	64.664	4.549	25.626
0.5	0.5	50	0.871295	0.857	28.355	1.353	12.143
1	1	100	0.886617	1.439	19.186	0.788	8.466

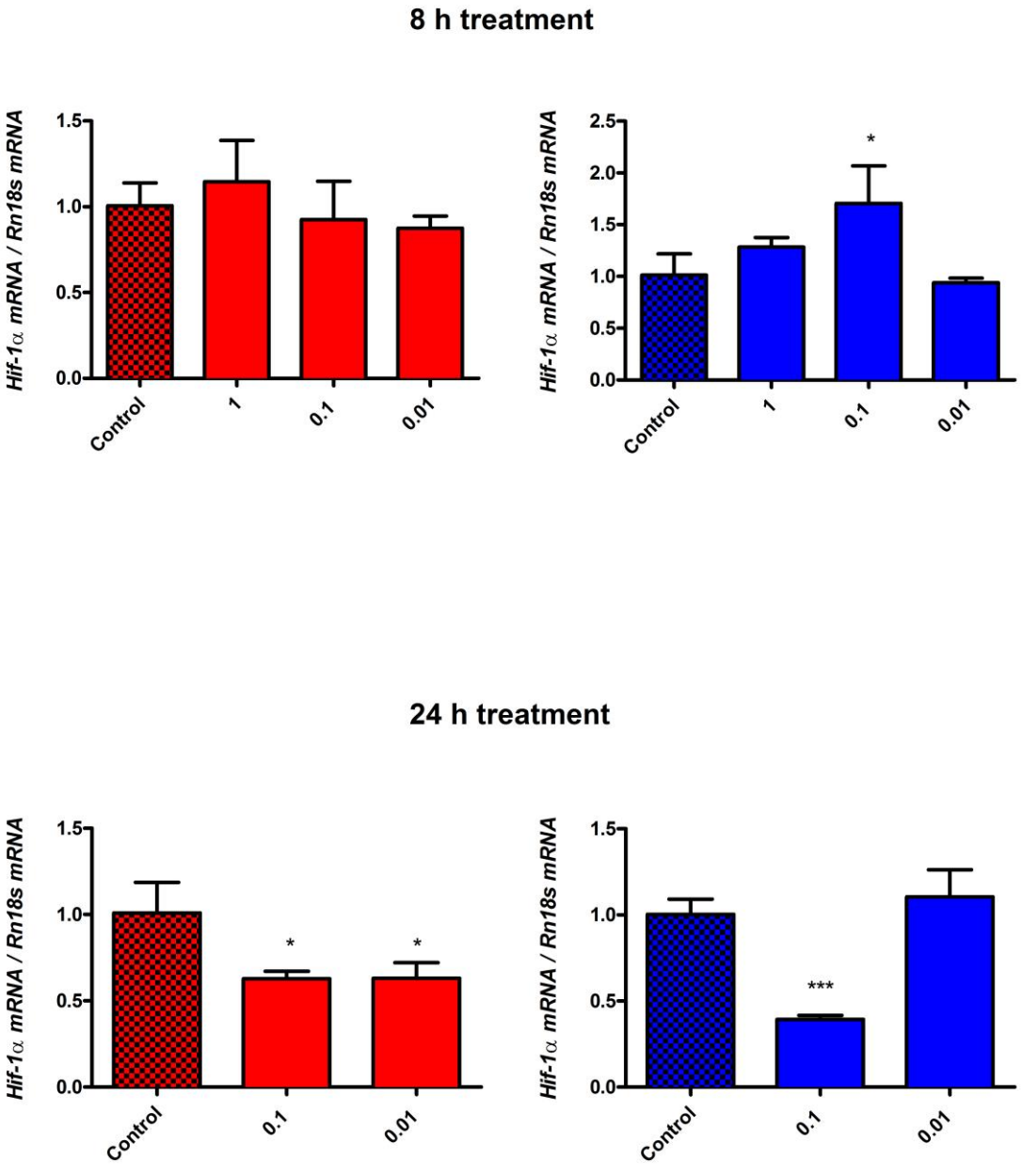
Supplementary Figure 1. Tumor cell growth inhibition by LMP-400 in normoxic and hypoxic conditions. Cell viability was measured by MTT assay after 48 hours of treatment with LMP-400 in hypoxic (1% O2, red curve) and normoxic conditions (21% O2, blue curve).



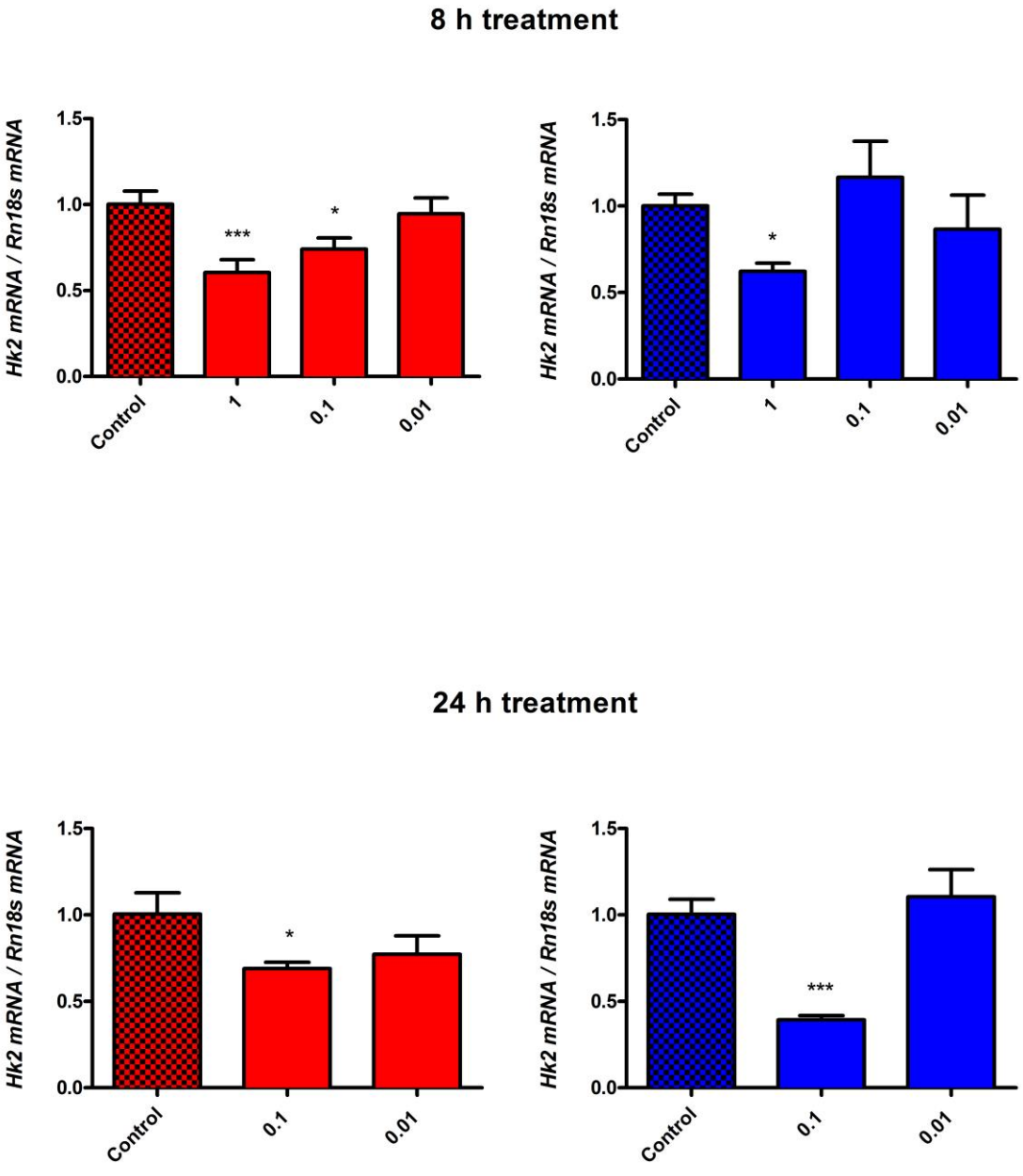
Supplementary Figure 2. Tumor cell growth inhibition by LMP-400 after short treatment periods. Cell viability was measured by MTT assay after 8- and 24-hour treatments with LMP-400 on established pheochromocytoma animal cell lines.



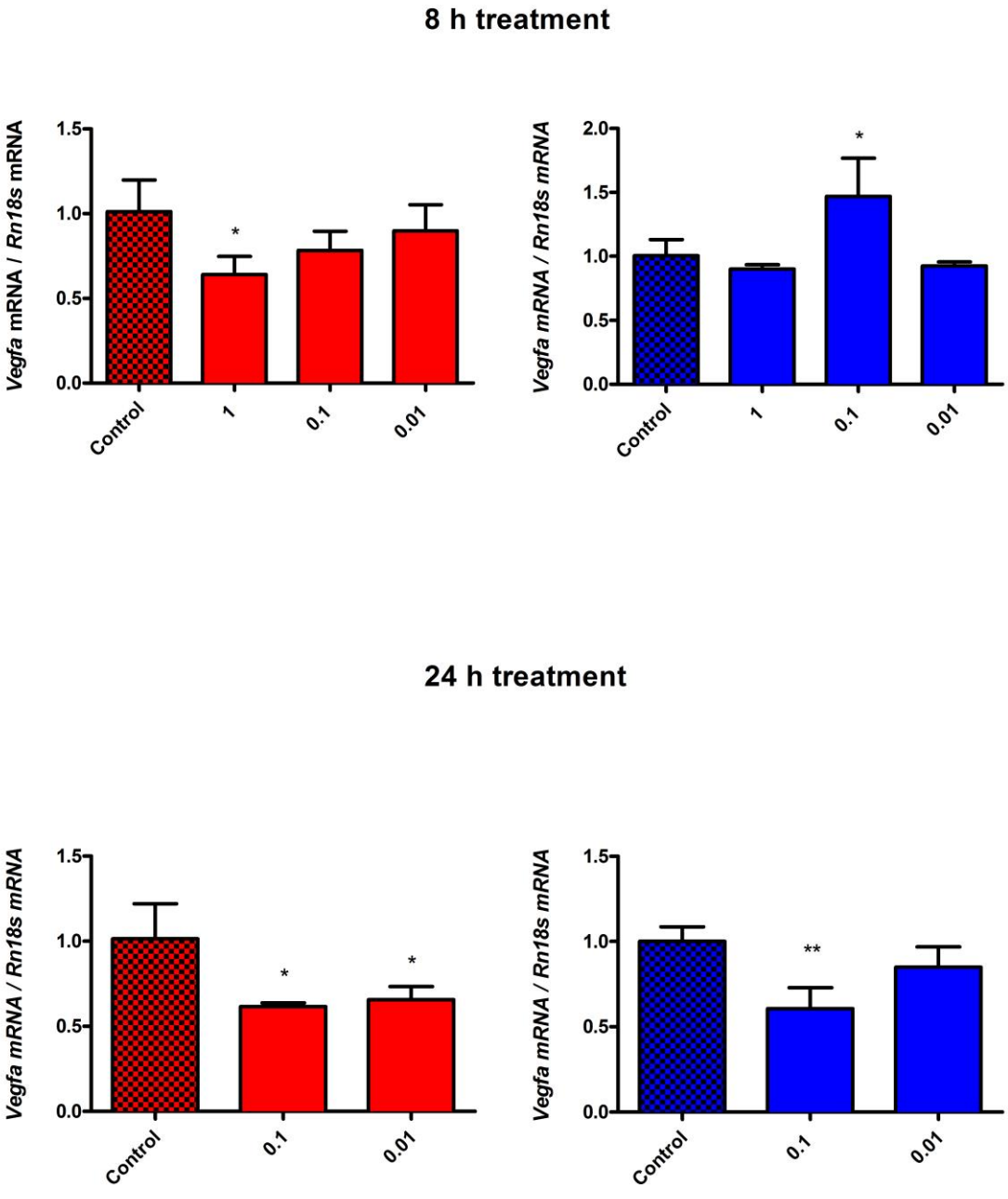
Supplementary Figure 3. Changes in Hif1α mRNA levels. Figure 3 depicts changes in mRNA levels of Hif1α upon 8- and 24-hour treatments with LMP-400 in hypoxic (1% O2, red column) and normoxic conditions (21% O2, blue column).



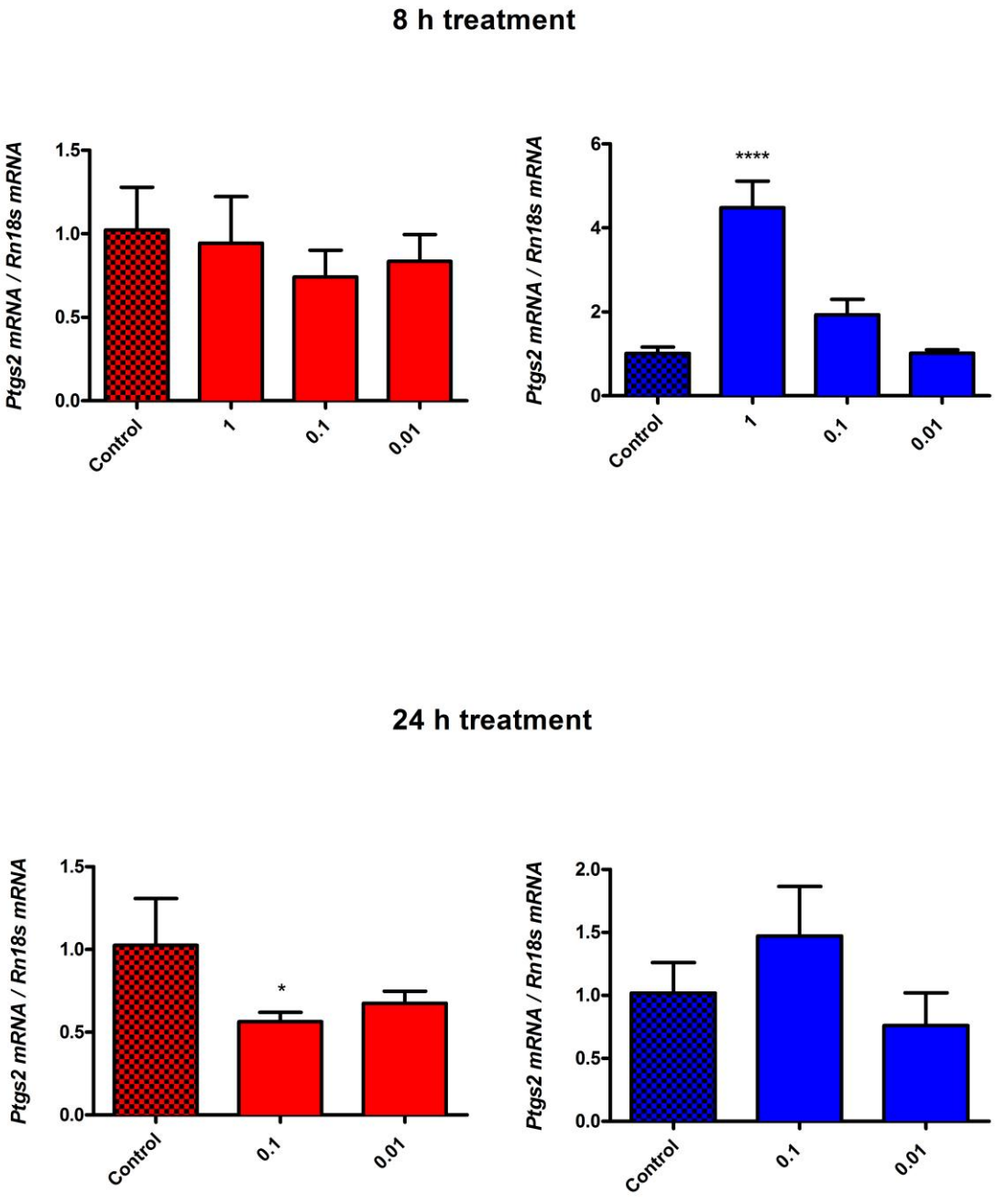
Supplementary Figure 4. Changes in Hk2 mRNA levels. Figure 4 depicts changes in mRNA levels of Hk2 upon 8- and 24-hour treatments with LMP-400 in hypoxic (1% O2, red column) and normoxic conditions (21% O2, blue column).



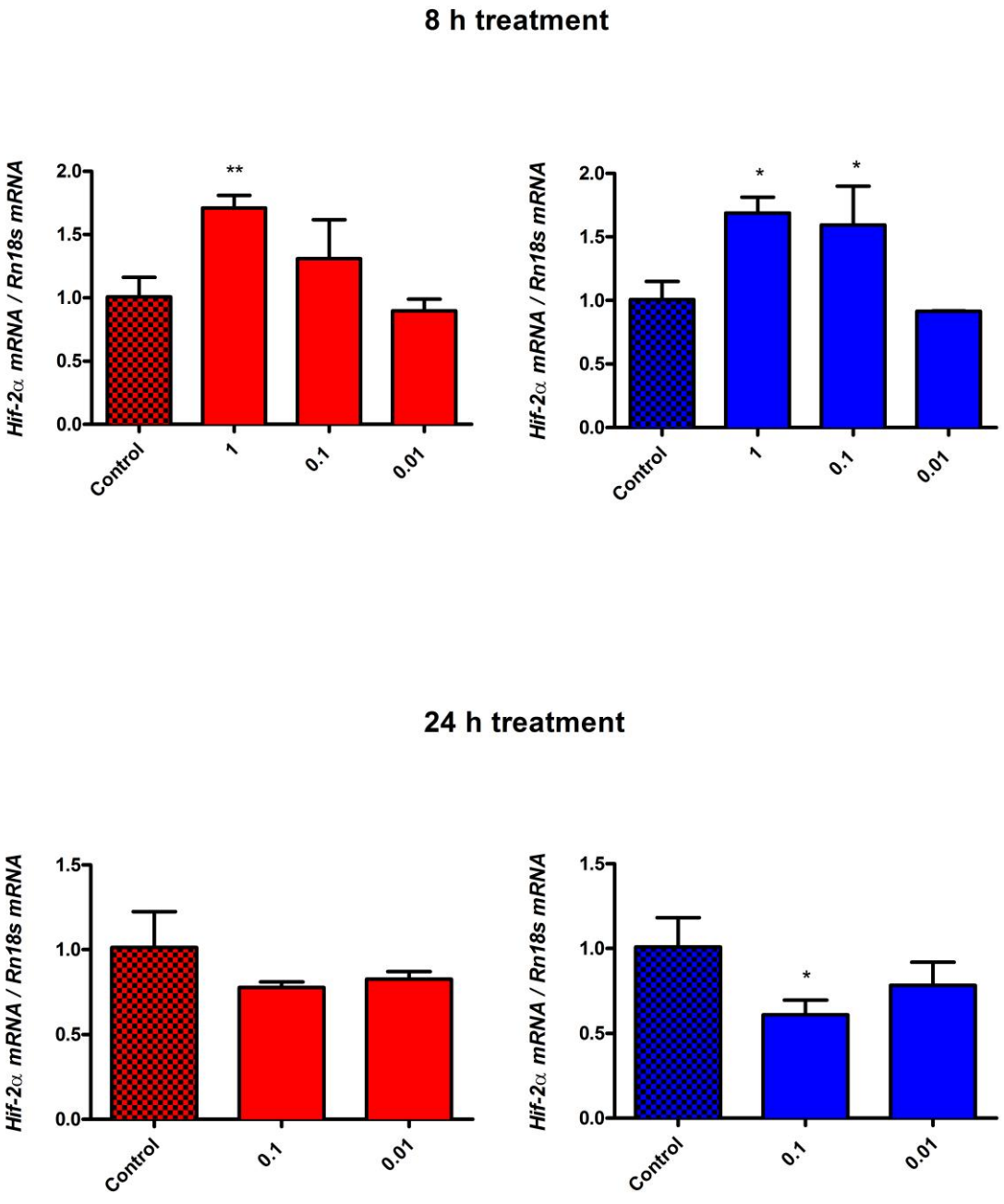
Supplementary Figure 5. Changes in Vegfa mRNA levels. Figure 5 depicts changes in mRNA levels of Vegfa upon 8- and 24-hour treatments with LMP-400 in hypoxic (1% O2, red column) and normoxic conditions (21% O2, blue column).



Supplementary Figure 6. Changes in Ptgs2 (Cox-2) mRNA levels. Figure 6 depicts changes in mRNA levels of Ptgs2 (Cox-2) upon 8- and 24-hour treatments with LMP-400 in hypoxic (1% O2, red column) and normoxic conditions (21% O2, blue column).



Supplementary Figure 7. Changes in Hif2α mRNA levels. Figure 7 depicts changes in mRNA levels of Hif-2α upon 8- and 24-hour treatments with LMP-400 in hypoxic (1% O2, red column) and normoxic conditions (21% O2, blue column).



Pdf version of the published article

IV. List of Shortcuts

18F-FDA	18F-fluorodopamine
18F-FDG	18F-fluorodeoxyglucose
18F-FDOPA	18F-3,4-dihydroxyphenylalanine
ACUC	Animal Care and Use Committee
CVD	Cyclophosphamide, Vincristine, Dacarbazine
FDA	Food and Drug Administration
GRADE	Grading of Recommendations, Assessment, Development, and Evaluation
Hif-2α	gene encoding hypoxia-inducible factor 2α
ISP	International Symposium on Pheochromocytoma
LCMS/MS	Liquid Chromatography tandem Mass Spectrometry
MEN	Multiple Endocrine Neoplasia
MN	Metanephrine
NCI	National Cancer Institute
NIH	National Institutes of Health
NMN	Normetanephrine
PGL	Paraganglioma
PHEO	Pheochromocytoma
RDBP	Negative Elongation Factor Complex Member E
ROC	Receiver Operating Characteristic
SDHB	Succinate Dehydrogenase Subunit B
URL	Upper Reference Limit
VMA	Vanillylmandelic acid
WHO	World Health Organization

Articles with IF, with direct connection to the topic of doctoral thesis as a co-author, Pdf versions of the published articles

....

Article with IF, without direct connection to the topic of doctoral thesis as a co-author, Pdf version of the published article