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## How far have we come and how much further still to go?

**Jacques Lenders**[1]

*1. Radboud University Nijmegen Medical Centre, Netherlands*

Clinical care of pheochromocytoma/paraganglioma (PPGL) has greatly improved over the last decades due to tremendous advances in biochemistry, imaging, genetics and therapeutics. From the historical perspective the names of Manger, Gifford, Crout and Sjoerdsma deserve recognition for their seminal work on PPGLs. Prior to 1964 two thirds of all cases were discovered at autopsy. While most patients now are diagnosed while living, some still die with an undiscovered PPGL despite the availability of highly effective diagnostic and therapeutic modalities. Lack of awareness, ignorance of clinical clues, and poor post-surgical follow-up are to blame for this.

In recent years improved analytical technologies, enabling a more detailed understanding of catecholamine metabolism, has resulted in a paradigm shift in laboratory testing from catecholamines to metanephrines. The precise diagnostic and theranostic role of promising new functional imaging agents beyond conventional MIBG need to be defined. Modern surgical and sophisticated anesthetic management of PPGLs has resulted in a near zero perioperative mortality but reliable post-surgical prediction of metastatic disease is currently still impossible despite the availability of several risk stratification scores.

Revolutionary advances in molecular biology and genetics have made it clear that PPGLs have the richest hereditary background of all tumors with more than one third due to a germline mutation. The evolving role of somatic mutations in apparent sporadic PPGLs for clinical practice will be established. Growing evidence shows that hereditary PPGLs are characterized by distinct clinical presentations with differences in biological behavior reflecting underlying mutations. Advanced integration of genomics and metabolomics will further contribute to improved personalized diagnostics, treatment and surveillance.

A major outstanding clinical problem continues to be the lack of effective curative treatment of metastatic PPGLs. A concerted multidisciplinary approach of basic and clinical research, using relevant in vitro en in vivo models for identification of therapeutic targets for development of new drugs to be evaluated in well-designed multicenter clinical trials, is crucial for improving the prognosis of these patients.

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## The TCGA data on phaeochromocytoma and paraganglioma

**Lauren Fishbein**[1]

*1. Department of Medicine/Division of Endocrinology, Metabolism and Diabetes, University of Colorado School of Medicine, Aurora, CO, United States*

The Cancer Genome Atlas (TCGA) sought to identify molecular alterations and classifications, as well as clinically-associated molecular markers of different cancer types, including pheochromocytomas and paragangliomas (PCCs/PGLs). The PCC/PGL TCGA study analyzed 173 primary tumors across several genomic platforms including whole exome sequencing, mRNA sequencing, miRNA sequencing, methylome and reverse phase protein array. This large integrative genomic study offered novel insights into tumorigenesis and aggressive disease. Most PCC/PGL contained a single driver, either as a germline or somatic mutation or fusion gene. This study was the first to identify a recurrent fusion gene in PCC/PGL, which involved *MAML3* and lacked the *MAML3* canonical NOTCH binding site. In fact, the tumors with the fusion gene did not have upregulation of NOTCH signalling, but instead showed increased Wnt and Hedgehog signalling, suggesting a novel pathway in PCC/PGL tumorigenesis. Furthermore, the presence of the *MAML3* fusion gene was associated with clinically aggressive disease. Germline *SDHB* and somatic *ATRX* mutations as well as Ki-67 index were reinforced as markers of clinically aggressive disease. In addition, TCGA data confirmed the kinase and pseudohypoxia expression subtypes and identified two novel subtypes, Wnt-altered and cortical admixture. The Wnt-altered subtype tumors were sporadic, associated with clinically aggressive disease, and contained all the tumors with the *MAML3* fusion and three of four tumors with somatic *CSDE1* mutations, a new somatically mutated gene in association with PCC/PGL. Interestingly, the hypermethylated pseudohypoxia subtype, associated with clinically aggressive disease, contained 74% of the genome-doubled tumors, most of which had *VHL* or *EPAS1* mutations. One limitation to the study is the small number of metastatic tumors in the cohort. Overall, the PCC/PGL TCGA data describe four molecular subtypes with a low genomic alteration rate but diverse driver alterations serving as novel biomarkers for clinically aggressive disease and potential targets for therapy.

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## When we have all had our genomes sequenced

**Emma Duncan**[1]

*1. Queensland University of Technology, Brisbane, QLD, Australia*

coming soon

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## Stem cells and neural crest differentiation

**Quenten Schwarz**[1], **Rachael Lumb**[1], **Christiana Ruhrberg**[2]

*1. Centre for Cancer Biology, Adelaide, SA, Australia*

*2. UCL Institute of Ophthalmology, University College London, London, United Kingdom*

The adrenal gland is composed of a medulla and cortex that secrete adrenaline and corticosteroids into the systemic circulation to maintain physiological homeostasis and enable the autonomic stress response. Connections between central nervous system preganglionic sympathetic neurons and chromaffin cells in the adrenal medulla are essential for this stress response. However, the mechanisms that enable the formation of functional circuits between preganglionic neurons and chromaffin cells are currently unknown. By combining lineage tracing, gene expression studies and the analysis of engineered mouse mutants, we have found that axon innervation of the adrenal primordia precedes chromaffin cell differentiation, and that the neural crest cell precursors of chromaffin cells colonise the adrenal medulla in unison with axonal innervation. We further found that mice with axon guidance defects due to a lack of neuropilin (NRP) 2 or its secreted ligand SEMA3F have defective innervation of the adrenal primordia and ectopically positioned chromaffin cells. Moreover, we found that SEMA3A cooperates with SEMA3F to guide preganglionic axons and chromaffin cell precursors into the adrenal primordia. These observations demonstrate that sympathoadrenal neural crest cells are guided to the adrenal primordia along the axons with which they will form functional connections to lay an anatomical foundation for the central regulation of the autonomic stress response.

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## What do patients expect from us?

**Simone Leyden**[1], **Allen Wilson**[2]

*1. Unicorn Foundation, Blairgowrie, VIC, Australia*

*2. Pheo Para Troopers, New York, USA*

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## Beyond Paragangliomas: Getting the GIST of Syndromically Associated Tumors

**Arthur S Tischler**[1], **James F Powers**[1], **Brent Cochran**[1], **James Baleja**[1]

*1. Tufts University School of Medicine and Tufts Medical Center, Boston MA, USA*

SDH-deficient GISTs are rare but sometimes lethal tumors that can affect patients with paraganglioma syndromes. These tumors do not harbor the c-Kit or PDGF mutations typical of other GISTs and are therefore not amenable to typical GIST treatments with tyrosine kinase inhibitors. They can grow unpredictably, metastasize to lymph nodes and exhibit extensive local invasion. As with PGLs, there is currently no cure other than complete surgical excision, and there is a paucity of experimental models. The lifetime risk of developing SDH-deficient GIST in patients with *SDHB* mutation is still uncertain. However, the tumors accounted for ~3% of all GISTs and 5% of gastric GISTs in an unselected adult population.

We now have in our laboratory the first Patient Derived Xenograft (PDX) and cell culture model for basic and pre-clinical studies of SDH-deficient GIST. The model, which we have named the Ian GIST model, is derived from a gastric GIST that arose in a young man with a hereditary *SDHB* mutation and family history of multiple paragangliomas. Despite a remarkable multi-institutional translational research effort aimed at developing an effective treatment ("the Ian GIST Project"), the entire clinical course before the patient died was approximately 3 years. In addition to absence of SDHB protein expression and loss of SDH activity, the tumor was found to harbor a somatic KRAS G12D mutation, possibly contributing to its aggressive behavior.

It is generally believed that use of early passage PDX models for preclinical drug testing predicts clinical outcomes better than cell lines or grafts derived from cell lines. However, cell lines are needed for mechanistic research. Further, PDX models, like cell lines, are known to exhibit phenotype drift over multiple consecutive passages. In view of these concerns we have adopted a multi-tiered approach to this model, tracking morphology, transcriptomic and metabolomic profiles in consecutive *in vivo* passages and in transition to cell cultures. The pathology of the patient-derived xenografts is very similar to that of the patient's surgically resected tumor except for somewhat increased proliferation in the fifth mouse passage. The tumor expresses GIST markers including c-Kit (also known as CD117, the receptor tyrosine kinase for stem cell growth factor) but is of an unusually high histologic grade compared to most GISTs. An important difference from primary paragangliomas and preliminary PDX models of paraganglioma is an extremely high proliferative fraction in the GIST, as assessed by Ki 67 labeling. This is consistent with the rapid clinical course and fatal outcome of the tumor, in contrast to the relatively slow growth and prolonged clinical course of many paragangliomas. This difference in proliferation between tumor types would likely result in greatly different responses to many types of cytotoxic drugs. At the same time, electron microscopy of the GIST cells shows extensive evidence of autophagy and mitochondrial stress, suggesting possible vulnerabilities to new types of drugs targeting those properties.

This GIST model is important for two reasons. First, it is a unique human-derived model for a specific type of tumor that occurs in patients with hereditary *SDHB* mutations. Although the basic metabolic defect in all SDH-deficient tumors is similar, different tumor types are likely to be affected by that defect in different ways because of intrinsic differences in their cells of origin. Second,

because SDH-deficient GIST does have similarities to other SDH-deficient tumors, information gained from studying this model might also lead to increased understanding of and improved treatments for other tumor types.

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## Succinate dehydrogenase (SDH) deficient neoplasia

**Anthony Gill**[1, 2, 3]

*1. University of Sydney, Sydney, NSW, Australia*

*2. Department of Anatomical Pathology, Royal North Shore Hospital , St Leonards, NSW , Australia*

*3. Cancer Diagnosis and Pathology Research Group, Kolling Institute of Medical Research , NSW, Australia*

The succinate dehydrogenase (SDH) complex is a key respiratory enzyme composed of four subunits SDHA, SDHB, SDHC and SDHD. Remarkably immunohistochemistry for SDHB becomes negative whenever there is bialleic inactivation of any component of SDH which is very rare in the absence of syndromic disease. Therefore loss of SDHB immunohistochemistry serves as a marker of syndromic disease, usually germline mutation of one of the *SDH* subunits. Tumours which show loss of SDHB expression are termed *succinate dehydrogenase deficient*. In addition to loss of SDHB, tumours associated with *SDHA* mutation also show loss of SDHA expression. The identification of succinate dehydrogenase deficient neoplasm facilitates genetic testing and risk reduction strategies.

15% of pheochromocytoma and paraganglioma (PHEO/PGL) are associated with germline *SDH* mutation and therefore SDH deficient. We recommend screening SDHB immunohistochemistry for all PHEO/PGL.

SDH deficient Gastrointestinal Stromal Tumours (GISTs) show distinctive features including absent *KIT*/*PDGFRA* mutations (but positive staining for cKIT and DOG1), virtually exclusive gastric location, lobulated growth, multifocality, a prognosis not predicted by size and mitotic rate, metastasis to lymph nodes and primary resistance to imatinib therapy. 30% are associated with *SDHA* germline mutation. 50% are associated with *SDHC* epimutation (post zygotic promoter hypermethylation) - the hallmark of the syndromic but non-hereditary Carney Triad (SDH deficient GIST, SDH deficient paraganglioma and pulmonary chondroma).

SDH deficient renal carcinoma is newly recognised under the WHO 2016 classification and usually demonstrates characteristic morphology including vacuolated eosinophilic cytoplasmic and cytoplasmic inclusions. SDH deficient renal carcinoma is particularly associated with *SDHB* mutation, although *SDHC* and *SDHA* mutation occur. SDH deficient pituitary adenomas are recognised but appear to be the least common SDH deficient neoplasm.

**Table 1: SDH abnormalities in Phaeochromocytoma and Paraganglioma**

| Syndrome | Gene | Chromosome | Inheritance | Maternally Imprinted | Frequency | Penetrance | Gender distribution | Adrenal | Abdomen | Thorax | Head & neck | Metastasis | GIST | Renal cell carcinoma | Pituitary adenoma | Pulmonary Chondroma |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PGL1** | *SDHD* | 11q23 | Autosomal Dominant | Yes | Common | | Equal | + | + | rare | +++ | < 5% | + | + | + | - |
| **PGL2** | *SDHAF2* | 11q12.2 | Autosoaml Dominant | Yes | Very rare | | Equal | - | - | - | + + | low | - | - | + | - |
| **PGL3** | *SDHC* | 1q23.3 | Autosomal Dominant | No | Rare | Low | Equal | rare | rare | rare | + + (particularly carotid body) | low | + | ++ | + | - |
| **PGL4** | *SDHB* | 1p36.1-p35 | Autosomal Dominant | No | Common | High | Equal | + | +++ | ++ | ++ | 31-71% | + | +++ | + | - |
| **PGL5** | *SDHA* | 5p15 | Autosomal Dominant | No | Rare | Very low | Equal | - | + | + | + | ? | +++ (30% of SDH deficient GISTs) | + | ++ | - |
| **Carney triad** | *SDHC promoter hypermethylation* | 1q23.3 | Not hereditary | No | Very rare | N/A | Female predominant | - | ++ | - | + | ? | ++++ (50% of SDH deficient GIST) | - | - | ++ |

**Table 2: Phenotype-Genotype correlations in SDH deficient neoplasia**

| Tumour | SDHA mutation | SDHB mutation | SDHC mutation | SDHD mutation | SDHAF2 mutation | SDHC promoter hypermethylation (Carney Triad) |
|---|---|---|---|---|---|---|
| Pheo/PGL | + | +++ | ++ | +++ | + | ++ |
| GIST | +++ | + | + | + | - | +++ |
| Renal Carcinoma | + | +++ | + | +/- | - | - |
| Pituitary Adenoma | ++ | + | + | + | - | - |
| Pulmonary Chondroma | - | - | - | - | - | +++ |

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## Role of microenvironment in Pheochromocytoma/Paraganglioma growth and invasiveness

**Massimo Mannelli**[1]

*1. University of Florence, Sesto Fiorentino, FI, Italy*

Microenvironment is an active player in solid tumor biology. Tumor cells establish a functional and metabolic crosstalk with tumor cells favoring their survive and growth in a hostile environment. Such an interplay also favors tumor aggressiveness and spread.

Pheochromocytomas and paragangliomas (PPGLs) are neural crest-derived tumors showing the highest rate of malignancy when SDHB mutated.

Co-culturing the human neuroblastoma cell line SK-N-AS with fibroblasts, as representative of microenvironment, a reciprocal effect between the two type of cells changes their metabolic pattern as well as tumor cell growth and invasion potential. SDHB silencing increases these effects. When using a mouse metastatic pheochromocytoma cell line (MTT), a similar crosstalk with fibroblasts was observed, with minor differences in the metabolic consequences while the increase in tumor cell migration/invasion were confirmed and found further enhanced by SDHB silencing. These studies strongly support a role for the microenvironment in sustaining tumor growth and invasiveness. These effects are further enhanced in SDHB silenced cells. The comprehension of the factors that specifically increase the invasiveness of SDHB silenced cells might suggest potential therapeutic targets to block or slow the progression of SDHB related metastatic PPGLs.

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## Characterization of cultured multipotent human fetal neural crest-derived cells

**Elena Rapizzi[1], Cristiana Deledda[1], Susanna Benvenuti[1], Gabriella B Vannelli[1], Rossella Fucci[1], Paola Luciani[1], Benedetta Fibbi[1], Corinna Giuliani[1], Benedetta Mazzanti[1], Daniele Bani[1], Daniele Guasti[1], Michaela Luconi[1], Milena Pantaleo[2], Letizia Canu[1], Tonino Ercolino[1], Alessandro Peri[1], Massimo Mannelli[1]**

*1. University of Florence, Florence, ITALY, Italy*

*2. Azienda Ospedaliera Meyer, Florence, Italy*

Neural Crest Cells are a transient embryonal population originating from the neural ectoderm border. NCCs migrate and generate differentiated cell types, including neurons and glia of the peripheral nervous systems, chromaffin cells, melanocytes, skeletal and connective components of the head. Alteration of the NCCs function causes developmental and congenital defects. Human NCCs are difficult to obtain and current knowledge derives mainly from animal models. Recently, human cell models have been obtained by embryonic stem cell or induced pluripotent stem cells but, up to date, *in vitro* data originate from engineered and/or tumoral cell lines.

In this study we developed a new human neural crest-derived cell model expressing neural crest specifier genes. NCC differentiated towards lineages originated *in vivo* by NCCs, induced a mature phenotype in a neuronal model acting like Schwann precursors, possessed migration activity and acquired a sympatho-adrenal phenotype.

In conclusion, this unique cell model provides a potential tool for studying the physiopathology of human fetal development and tumorigenesis. The effects caused on metabolism, functions and genetic profile of these cells by silencing pheochromocytoma/paraganglioma susceptibility genes, especially *SDHB*, may provide additional information on the pathogenesis of these tumors.

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## Towards inhibition of activated HIF-2 and patient-derived xenograft (PDX) PPGL models

**Sven Påhlman[1], Caroline Wigerup[1], Camilla Persson[1], Daniel Bexell[1], Noemie Braekeveldt[1], Javan Esfandyri[1]**

*1. Lund University, Lund, SWEDEN, Sweden*

Gene expression signatures of paragangliomas and pheochromocytomas (PPGLs) reveal two main clusters where tumors with SHDx and VHL mutations belong to a cluster which has a gene signature associated with hypoxic signaling or pseudohypoxia. Mutations in either *VHL* or *SDH* genes lead to the stabilization of hypoxia-inducible factor 1a and 2a (HIF-1a and HIF-2a) and occasionally, HIF2A/EPAS1 is activated through a mutation in the VHL-recognition amino acid. Recently, a selective HIF-2 inhibitor, PT2385 was reported. PT2385 binds to a HIF-2a unique protein pocket in the PAS-B domain, and thus, prevents the HIF-2a-ARNT dimerization and the formation of an active HIF-2 transcription complex. We synthesized PT2385 and evaluated its effect(s) on clear cell renal carcinoma cells (ccRCC) and neuroblastoma cells, cell types with an active HIF-2 at normoxic or hypoxic conditions. While PT2385 inhibited the expression of HIF-2 driven genes like VEGF in HIF-2a expressing CCRCC cells with lost VHL and HIF1A expression, VHL mutated ccRCC cells with intact HIF1A and HIF2A did not respond equally well to PT2385 treatment. Neuroblastoma PDX-derived cell lines with high HIF-2a expression at normoxia as well as serum-cultured established cell lines with hypoxia-stabilized HIF-2a did not respond to PT2385 by down-regulation of HIF-2 driven genes like VEGF. In contrast, siHIF2A, which knocks down HIF-2a expression, diminishes the expression of HIF-2 down-stream target genes. Putative models explaining our data will be discussed. We will also report our ongoing efforts to establish orthotopic human PPGL-PDXs and describe the network of clinicians we collaborate with to get relevant tumor tissue for these studies.

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## Zebrafish mutants in the von Hippel-Lindau tumor suppressor display a normetanephrine dominant phenotype

**Peter MT Deen[1], Selma Waaijers[1, 2], Antonius E van Herwaarden[2], Rachel Giles[3], Henri JLM Timmers[4]**

*1. Physiology, Radboud Univ. Med. Center, Nijmegen, Netherlands*

*2. Laboratory Medicine, Radboud Univ. Nijmegen Med Center, Nijmegen, Gelderland, The Netherlands*

*3. Nephrology and hypertension, University Medical Center Utrecht, Utrecht, The Netherlands*

*4. Internal Medicine, Radboud Univ. Nijmegen Med Center, Nijmegen, The Netherlands*

**Introduction:** Zebrafish *vhl* mutants develop key aspects of the human disease condition, including activation of the hypoxia-inducible factor (HIF) signaling pathway, polycythemia, excessive neovascularization, macular edema, and pronephric abnormalities.[1] Whether the human *VHL*-related pheochromocytoma/paraganglioma (PPGL) phenotype is replicated in *vhl* mutant zebrafish has not been investigated. Our aim was to develop a method to measure catecholamines and metanephrines in zebrafish and to investigate the presence of features of PPGL.

**Methods:** liquid chromatography/mass spectrometry was used to measure metanephrines in zebrafish of lysates of larvae and in (urine containing) medium they were swimming in. Concentrations of metanephrines were compared between *vhl*-/- and wild-type (wt) zebrafish.

**Results:** Protocols for optimal sample preparation and (nor)metanephrine assays were established for both fish lysates and swimming medium. In lysates of 30 5-days-post-fertilization wt zebrafish larvae, the mean levels of metanephrine and normetanephrine were 0.010±0.001 (SEM and 0.036±0.002 pmol/larva, respectively. In 30 *vhl*-/- zebrafish larvae, normetanephrine levels were 2-fold higher (0.056±0.01; P<0,01), whereas metanephrine levels were >2 (0.004±0.001; P<0,001). In the swimming water, metanephrins were undetectable, while in medium of *vhl*-/- larvae the normetanephrine levels were 2,5-fold increased () as compared to wt (1.093±0.115 versus 0.429±0.049 pmol/larva; P<0,01). No obvious tumors of the fish's 'adrenal' gland were observed.

**Conclusion:** We have established a method for the quantification of (nor)metanephrines in zebrafish and *Vhl* knock-out larvae display a normetanephrine dominant phenotype despite absence of obvious PPGL-like tumors. As such, *vhl* zebrafish represent a unique *in vivo* model for studying human VHL genotype–phenotype correlations, including (features of) PPGL.

1. Van Rooijen E, et al. Zebrafish mutants in the von Hippel-Lindau tumor suppressor display a hypoxic response and recapitulate key aspects of Chuvash polycythemia. Blood. 2009 Jun 18;113(25):6449

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## Synthetic lethal testing in a mouse embryonic fibroblast model of SDH-loss human familial paraganglioma

**Oksana Hamidi[1], John A Smestad[1], Lin Wang[1], Molly H Nelson Holte[1], L. James Maher III[1]**

*1. Mayo Clinic, Rochester, MN, United States*

**Background:**

Succinate dehydrogenase (SDH)-loss paraganglioma (PGL), a rare neuroendocrine tumor derived from autonomic paraganglia, is a model for tumorigenesis driven by metabolic derangement. SDH is an enzyme of the tricarboxylic acid (TCA) cycle that oxidizes succinate to fumarate. Accumulation of succinate in the setting of SDH loss is believed to drive PGL tumorigenesis via activation of pseudohypoxic signaling and induction of hypermethylation of histones and DNA. SDH loss may obligate PGL tumor cells to rely upon otherwise non-essential metabolic pathways for survival. Previous evidence has suggested that SDH-deficient cells rely on lactate dehydrogenase A (LDHA) for regeneration of NAD+, raising the possibility that LDH inhibition might be selectively toxic to SDH-loss cells. Likewise, SDH loss may induce dependence on pyruvate carboxylase (PCx) for uptake of extracellular pyruvate and increased aspartate synthesis. In the current work, we investigate the effects on cell viability of SDH loss in the context of inhibition of putative PGL therapeutic targets, LDHA and PCx.

**Methods:**

We developed an immortalized mouse embryonic fibroblast (iMEF) cell line with doxycycline-triggered conditional disruption of SDHC alleles. Using homozygous SDH loss model and a heterozygous control, we characterized the time-course of SDHC gene loss and protein loss following doxycycline treatment. We then performed lentiviral short hairpin RNA (shRNA) knockdown of LDHA and PCx in SDHC-loss and control iMEFs and assessed apoptosis induction via a fluorescence image-based caspase activity assay. Similarly, we assessed differences in cell viability between SDH-loss and control iMEF lines following exposure to known pharmacologic inhibitors of LDH.

**Results:**

We present quantitative apoptosis data showing that LDHA and PCx loss are synthetically lethal with SDH loss. These results are compared with new data testing differential vulnerability of SDH-loss iMEFs to small molecule inhibitors. SDH-loss PGL may be selectively vulnerable to growth inhibition by non-cytotoxic metabolic inhibitors.

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## New PET modalities for pheochromocytoma and paraganglioma

**Karel Pacak[1], David Taieb[2]**

*1. NICHD, Bethesda, MD, United States*

*2. Department of Nuclear Medicine, Aix-Marseille University, Marseille, France*

Nuclear medicine has emerged at the forefront of precision medicine. Positron emission tomography (PET) has a unique potential that enables noninvasive visualization of a wide variety of physiological and pathological processes at the metabolic and molecular level. The presence of catecholamine synthesis and metabolism, norepinephrine and amino acid transporter systems, somatostatin receptors and GLUT transportrs, and the presence of stromal cells on these tumors guided physicians and scientists to utilize various available radiopharmaceuticals in the assessment of pheochromocytoma and paraganglioma (PPGL). Four emerging PPGL imaging phenotypes have been introduced to these tumors: 1. Pseudohypoxia $^{18}$F-FDG imaging phenotype; 2. Stromal cell-succinate $^{18}$F-FDG imaging phenotype; 3. Catecholamine metabolism-imaging phenotype; and 4. Somatostatin receptors-imaging phenotype. Thus, now nuclear imaging-based disease phenotyping provides a better understanding of PPGLs with these imaging phenotypes: a pseudohypoxia $^{18}$F-FDG imaging phenotype common to all cluster 1 tumors; a stromal cell-succinate $^{18}$F-FDG imaging phenotype linked to the presence of succinate dehydrogenase deficiency and due to the hormone-like effect of succinate on $^{18}$F-FDG uptake; a catecholamine metabolism-imaging phenotype dependent on both tumor content of catecholamines and the secretion of their metabolites, thereby providing information on PPGLs functional differentiation; and somatostatin receptors-imaging phenotype that further characterizes PPGLs that now enables the selection of patients for **peptide receptor radionuclide therapy using** somatostatin analogs (agonists and antagonists) that are labeled with therapeutic radioisotopes (such as $^{90}$Y,$^{177}$Lu or alpha emitters).

Furthermore, new trends in the use of $^{68}$Ga-labeled somatostatin analogs, 3DMR angiography sequences, and PET in combination with MR, HRMAS NMR spectroscopy for ex-vivo analysis of intact tissue are all becoming promising imaging and diagnostic approaches for these tumors. Moreover, the use of existing or new radiopharmaceuticals in the assessment of glycolysis, hypoxia, apoptosis, angiogenesis, and other tumor characteristics, including specific treatment targets, are being implemented in the evaluation of PPGLs and other tumors. Studies related to the mismatch between anatomical and functional imaging of PPGLs and other tumors, especially in the correct assessment of their therapeutic responses, proper follow up, and treatment continuation, are in progress.

In summary, nuclear endocrinology is becoming a reality and it is predicted to be pivotal in the "behavioral" assessment, proper treatment, and outcome of patients with PPGL and other endocrine tumors. It is expected that in the near future, initial PPGL functional imaging algorithms will be guided by the presence of specific gene mutations, tumor origin (sympathetic vs parasympathetic), and timing (age of the patient) of the development of these tumors reflecting ongoing initiative of personalized medicine.

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## The biological basis for PET imaging in PCC/PGL

**Rod J Hicks**[1]

*1. Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia*

Although anatomical imaging, including CT, MRI and ultrasound, is the most widely utilised approach for detection and staging of phaeochromocytoma and paraganglioma (PCC/PGL), there is increasing recognition of the utility of molecular imaging techniques. The first radiopharmaceutical developed for both the diagnosis and therapy of PCC/PGL was I-131 meta-iodo-benzylguanidine (MIBG), which is taken up by the norepinephrine transporter and stored in neurosecretory granules. Although traditionally labelled with the gamma-emitting radionuclides I-131 and, more recently, I-123, I-124 MIBG PET/CT imaging provides superior sensitivity for detection of small lesions compared to conventional gamma camera imaging due to higher spatial and contrast resolution. Use of PET/CT has the additional advantage of allowing quantitative analysis of tissue retention over time and thereby, estimation of potential radiation dose to tumour and normal tissues if radionuclide therapy is contemplated. Other catecholamine-like compounds that are suitable for PET imaging include C-11 hydroxyephedrine (HED), $^{18}$F-Meta Fluorobenzyl Guanidine (MFBG) and F-18 flurodopamine (FDA). Similarly, catecholamine precursors like $^{18}$F -L-fluoro-dihydroxyphenylalanine (FDOPA) have also been shown to detect some sites of disease. Like MIBG, these agents have relatively high specificity for Pheo/PGL but all have suboptimal sensitivity, especially for PGL of the head and neck region. Therefore, other aspects of tumour biology have been leveraged for diagnostic purposes or combined diagnostic and therapeutic ("theranostic") roles. Although non-specific, increased glycolytic metabolism has been shown to be a feature of many Pheo/PGL, especially those arising within the pseudo-hypoxia cluster, which leads to metabolic reprogramming. With the wide clinical availability of FDG PET/CT, this has become a practical alternative to MIBG for detection and staging of these tumours. Similarly, recognition of the frequent expression of somatostatin receptors (SSTR) on Pheo/PGL has also led to the use of radiolabelled somatostatin analogues (SSA), especially $^{68}$Ga-DOTA-octreotate for PET imaging. It is obviously not practical to use all these agents for evaluation of known or suspected Pheo/PGL and to a large extent the choice of radiotracer will be determined by local availability and regulatory approvals. Accordingly, there are no generally agreed algorithms that inform the use of these agents. Nevertheless, it is possible to establish rational guidelines regarding for whom, when and with which agent imaging should be considered using a combination of clinical and genomic characteristics of Pheo/PGL syndromes. Increasing understanding of the links between genotype and molecular imaging phenotype will strengthen investigation paradigms with an increasing focus on therapeutic options for patients.

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## In- and ex-vivo metabolic profiling of PPGL

**Henri Timmers**[1]

*1. Radboud University Medical Centre, Netherlands*

Pheochromocytomas and paragangliomas (PPGLs) are highly variable with respect to clinical presentation, endocrine phenotype, growth rate and metatastatic potential. This relates to a large genetic diversity and related pathways of tumorigenesis. By applying a multi-omics approach, profound abnormalities in tumor cell metabolism related to mitochondrial defects in a subset of PPGLs have been identified. Metabolic profiling of PPGL tumor tissues can be achieved by several techniques, including proton nuclear magnetic resonance spectroscopy and liquid chromatography- mass spectrometry. The underlying genotypes affect tumor cell metabolism beyond the Krebs cycle, impacting on purine/pyrimidine and amino acid metabolism, energy storage and oxidative stress response. Besides investigation of tumor tissues, metabolic profiling of PPGLs in patients can also be achieved non-invasively by 18F-fluorodeoxy glucose positron emission tomography and proton magnetic resonance spectroscopy. These *in-* and *ex-vivo* metabolomic approaches hold great promise for individual tumor characterization, thereby guiding tailor-made diagnostic and therapeutic strategies.

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## Role of $^{68}$Gallium Dotatate-PET/CT in pre-operative assessment of phaeochromocytoma and paraganglioma

**Matti L Gild**[1], **Jeremy Hoang**[2], **Edward Hsiao**[2], **Geoff Schrembri**[2], **Roderick Clifton-Bligh**[1]

*1. Endocrinology Department, Royal North Shore Hospital, St Leonards, NSW, Australia*

*2. Nuclear Medicine, Royal North Shore Hospital, St Leonards, NSW, Australia*

Diagnosis of paragangliomas (PGL) and phaeochromocytomas (PC) is challenging. The size, precarious location and functional heterogeneity of these rare neuroendocrine tumours subject patients to multiple investigations often yielding ambiguous results. Failure to diagnose can lead to preoperative morbidity and mortality as well as incomplete staging. Catecholamine assays are first line in assessment but are not helpful in non-functioning tumours and structural imaging with MRI and CT has limited specificity. PET/CT radiopharmaceuticals have been successfully employed to diagnose and stage neuroendocrine tumours. Somatostatin receptor imaging (SRI) agents have the highest sensitivity for these tumours, particularly the DOTA family of radiopharmaceuticals labelled with $^{68}$Gallium . We performed a retrospective analysis of all patients with PC and/or PGL at Royal North Shore Hospital between 2012 and 2017 who had preoperative SRI with $^{68}$Gallium Dotatate PET/CT. The number of cases included 58 PCs and 29 PGLs. SRI was performed in 39 PCs (67.2%) and in 28 (96.5%) PGLs. Sensitivity for this modality was 97.4% for PC and 95.8% for PGL. Metastases were found in 25.6% PCs vs 25% PGLs. Multifocal disease was identified in 3% PCs vs 33.3% PGLs. Incidental findings from SRI including thyroid nodules, parathyroid adenomas or incidental primary or metastatic neuroendocrine tumours were identified in 10.3% PC and 20.8% PGL. The application of SRI changed management in the majority of cases (79.5% PC and 55.2% PGL). We recommend that SRI scanning should be performed as first line to confirm the diagnosis of neuroendocrine tumours. Preoperative scanning should be performed in patients with phaeochromocytomas >5cm to exclude metastases and in all patients with hereditary paragangliomas syndromes to exclude multifocal disease. SRI using PET/CT is recommended for its superior quality, simple application lower cost and lower radiation than other commercially available analogues.

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## Impact of $^{123}$I-MIBG scintigraphy on clinical decision making in patients with pheochromocytoma and paraganglioma

**Dipti D Rao**[1], **Anouk van Berkel**[1], **Ianthe I Piscaer**[1], **William F Young**[2], **Lucinda L Gruber**[2], **Felix F Beuschlein**[3], **Ariadni A Spyroglou**[3], **Aleksander A Prejbisz**[4], **Katarzyna K Hanus**[4], **Graeme G Eisenhofer**[5], **Massimo M Manelli** [6], **Letizia L Canu**[6], **Timo T Deutschbein**[7], **Martin M Fassnacht**[8], **Jacques J.W.M. Lenders**[1, 5], **Irina I Bancos**[2], **Henri H.J.L.M. Timmers**[1]

*1. Department of Internal Medicine, Division of Endocrinology, Radboud University Medical Center, Nijmegen, The Netherlands*

*2. Division of Endocrinology, Diabetes, Metabolism, and Nutrition, Mayo Clinic, Rochester, USA*

*3. Medizinische Klinik und Poliklinik IV, Klinikum der Universität München,, Munich, Germany*

*4. Department of Hypertension, Institute of Cardiology, Warsaw, Poland*

*5. Department of Medicine III and Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav, Technische Universität Dresden, Dresden, Germany*

*6. Endocrinology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy*

*7. Department of Endocrinology and Diabetes, University Hospital Würzburg, Würzburg, Germany*

*8. Department of Endocrinology and Diabetes, University Hospital Würzburg, Würzburg, Germany*

***Background:*** Anatomical imaging with CT or MRI is regarded as first-choice modality for tumour localization in patients with pheochromocytoma and paraganglioma (PPGL). Functional imaging with $^{123}$I-labeled metaiodobenzylguanidine ($^{123}$I-MIBG) is widely used, but the added diagnostic value is unclear. We investigated the impact of $^{123}$I-MIBG scintigraphy on diagnosis and treatment of PPGL compared to anatomical imaging alone.

***Methods:*** In this retrospective international multicenter study we evaluated 340 patients with PPGLs (236 unilateral adrenal, 18 bilateral adrenal, 48 extra-adrenal, 12 multifocal, 26 metastatic) from seven centres. All patients underwent both CT and/or MRI and $^{123}$I-MIBG scintigraphy. Clinical data were obtained and local imaging reports were analysed centrally by two blinded independent observers. For each patient, tentative diagnoses based on CT/MRI alone, $^{123}$I-MIBG alone, CT/MRI plus $^{123}$I-MIBG were compared with the actual diagnosis as recorded in registries of the local centers. Results were analysed in relation to tumour location and size, biochemical phenotype, and genotype.

***Results****:* Diagnoses based on only CT/MRI and CT/MRI plus $^{123}$I-MIBG showed close concordance with actual diagnoses of 89 and 88.2% respectively (non-significant). In 3 out of 6 patients with false-negative results on CT/MRI, the diagnosis was corrected by $^{123}$I-MIBG (1 unilateral, 2 extra-adrenal). In 1 out of 30 patients with false-positive results on CT/MRI, diagnosis was corrected by $^{123}$I-MIBG. $^{123}$I-MIBG yielded false-positive lesions in 8 patients (7 unilateral, 1 extra-adrenal). Overall, $^{123}$I-MIBG resulted in an appropriate change in management for 4 patients (1%) and an inappropriate change for 9 patients (3%) (non-significant).

***Conclusion****:* The addition of $^{123}$I-MIBG scintigraphy to CT/MRI for localization of PPGL does not lead to more correct than incorrect changes in the diagnoses and treatment, even when $^{123}$I-MIBG scintigraphy is restricted to patients at risk for metastatic disease. However $^{123}$I-MIBG scintigraphy remains necessary for all patients with metastatic PPGL who qualify for $^{131}$I-MIBG radiotherapy.

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## Novel and less well known cluster 2-related PPGLs

**Patricia Dahia**[1]

*1. University of Texas Health Science Center, Texas, United States*

Molecular classification of pheochromocytomas and paragangliomas has provided insights into their underlying biology. The largest class of these tumors is represented by transcription Cluster 2, a designation that comprises a broad group of genetic lesions loosely unified by activation of kinase signaling events and protein translation. Components of this group involve well-known members of these signaling cascades, including kinase receptor (RET), activators (RAS) or suppressors (NF1 and MAX), but also less known components, such as transmembrane protein (TMEM127), core histone (H3F3A) and other drivers. Subclassification of this cluster has proven to be challenging, possibly reflecting the high degree of redundancy and cross-talk between constituents of these various signals. We will focus on the poorly understood components of this 'umbrella' class of pheochromocytomas and paragangliomas to discuss biological consequences of these mutations, implications of these findings for our understanding of the origin and development of these tumors, and the challenges ahead.

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## LESSONS FROM MTC APPLICABLE TO PHEOCHROMOCYTOMA

**Bruce Robinson[1], Diana E Benn[1]**

*1. The University of Sydney, Sydney, NSW, Australia*

There has been a great deal of clinical work done on the management of MTC in MEN2. In particular there have been several trials of tyrosine kinase inhibitors which have shown efficacy. In contrast, there have been only case reports of the use of these agents in PC. Sunitinib is reported to have some activity but other agents have not been systematically studied. Pheochromocytomas usually present in the third or fourth decade of life in patients with MEN2A. Generally the diagnosis is confirmed at the same time as or following the diagnosis of medullary thyroid carcinoma (MTC). Suspicion of the presence of MEN2 and in particular for pheochromocytoma is sometimes provided by the pattern of catecholamine (and catecholamine metabolite) excretion with raised epinephrine or epinephrine and norepinephrine being characteristic. Genetic testing is recommended to identify a heterozygous germline *RET* pathogenic mutation. Since 1993 and the identification of *RET* as the causative gene of multiple endocrine neoplasia type 2, genotype/ phenotype correlations have identified mutations likely to be associated with pheochromocytoma and with age specific disease penetrance of these mutations, as discussed in the revised ATA Guidelines[1.] Recent understanding of pheochromocytoma development includes the role of hypoxic and *RET* pathways, which may indicate a role for tyrosine kinase inhibitors. Trials of these agents should be undertaken and will require international collaboration.

1. 1. Wells et al., 2015 .Revised American Thyroid Association Guidelines for the Management of Medullary Thyroid Carcinoma. Thyroid 25:567-610.

**20**

## Contribution of targeted next-generation sequencing (NGS) to improve detection of germline and somatic mutations in pheochromocytomas and paragangliomas

**Nelly BURNICHON[2, 1], Laurene BEN AIM[2], Mathilde PADILLA-GIROLA[2], Caroline TRAVERS[2], Nelly LE POTTIER[2], Mathieu MADELAINE[2], Alexandre BUFFET[1], Judith FAVIER[1], Anne-Paule GIMENEZ-ROQUEPLO[2, 1]**

*1. Team 13, UMR970, Paris-Cardiovascular Research Center, INSERM, Paris, France*

*2. Georges Pompidou european Hospital, PARIS, 75, France*

**Context**

Since the first description of germline mutations in patients with paraganglioma or pheochromocytoma (PPGL), over 15 susceptibility genes have been identified. It is currently accepted that up to 60% of PPGL are associated with inherited or somatic mutation in these driver genes. Due to the high heritability, genetic testing has been recommended in all patients with PPGLs.

In this context, the use of Next-Generation Sequencing (NGS) represents a particularly relevant technique allowing a simultaneous screening of all PPGL genes of interest.

**Material**

A retrospective cohort including 202 tumour DNAs was analysed in order to validate the NGS protocol.

Since transfer to clinical practice, 461 leukocyte DNAs, 51 frozen-tumour DNAs and 10 FFPE-DNAs were sequenced.

**Methods**

We developed and optimized a custom amplicon-based NGS panel (SDH MASTR Plus v2 kit, Multiplicom) using a MiSeq (Illumina) instrument. The targeted panel included 17 genes (*SDHA, SDHAF2, SDHB, SDHC, SDHD, RET, VHL, NF1, TMEM127, EGLN1, EGLN2, FH, MAX, MDH2, EPAS1, ATRX* and *HRAS*). Primer design was compatible for DNA extracted from FFPE tissues. This panel allowed sequencing of 451 amplicons covering 182 exons. Data were analysed using SeqNext (JSI) and PolyDiag software. Presence of variant of interest was confirmed by Sanger sequencing. Pathogenicity of variants was evaluated by immunohistochemistry whenever possible.

**Results**

All mutations previously found using Sanger method[1] were confirmed by NGS (n=133). In the prospective study, 121 germline and 73 somatic variants of interest (class 3 to 5) were identified. Mutations were found in all tested genes. Functional studies were helpful for classification of 61 variants.

**Conclusion**

The use of targeted NGS approach improves the detection of mutations responsible for PPGL, especially somatic and mosaic mutations. Notably, the analysis of unselected PPGL tumour samples allows to clarify the implication of the different genes in development of sporadic PPGL.

1. Castro-Vega*, Letouzé*, Burnichon* et al. Multi-omics analysis defines core genomic alterations in pheochromocytomas and paragangliomas. Nat Commun. 2015.

**21**

## A Bayesian approach to determining penetrance of pathogenic variants in genes for hereditary pheochromocytoma/paraganglioma syndromes

**Diana (Dindy) Benn[1, 2, 3], Ying Zhu[4, 2, 3], Edward Kim[2, 3], Mathilda Wilding[4], Ashley Crook[4], Anne Louise Richardson[2], Catherine Luxford[2], Richard Tothill[5, 6], Emma L Duncan[7, 8, 9], Trish Dwight[2, 3], Arran Schlosberg[3], Katherine Tucker[10], Rod J Hicks[5, 6], Alison Trainer[5, 6], John Burgess[11], Rachel Susman[12], Lyndal Tacon[1, 2, 3], Bruce G Robinson[1, 2, 3], Anthony J Gill[13, 14], Michael Field[4], Roderick Clifton-Bligh[1, 2, 3]**

*1. Department of Endocrinology, Royal North Shore Hospital, St Leonards, NSW, Australia*

*2. Cancer Genetics Laboratory, Kolling Institute, Royal North Shore Hospital, Sydney, NSW, Australia*

*3. University of Sydney, Sydney, NSW, Australia*

*4. Northern Sydney Local Health District Familial Cancer Service, Royal North Shore Hospital, St Leonards, NSW, Australia*

*5. The Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia*

*6. The Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, Victoria, Australia*

*7. Queensland University of Technology, Brisbane, Queensland, Australia*

*8. Faculty of Medicine, University of Queensland, Brisbane, Queensland, Australia*

*9. Department of Endocrinology, Royal Brisbane and Women's Hospital, Brisbane, Queensland, Australia*

*10. Department of Clinical Genetics, Prince of Wales Hospital, Randwick, Sydney, NSW, Australia*

*11. University of Tasmania, Hobart, Tasmania, Australia*

*12. Genetic Health Queensland, Royal Brisbane and Women's Hospital, Brisbane, Queensland, Australia*

*13. RNSH, Glebe, NSW, Australia*

*14. Cancer Diagnosis and Pathology Group , Kolling Institute of Medical Research, Sydney, NSW, Australia*

Up until recently, determining penetrance of pathogenic variants required large observational cohort studies. The availability of large population cohort data in the Exome Aggregate Consortium (ExAC) now makes it possible to use a Bayesian approach to calculate penetrance, in that population frequencies of pathogenic germline variants should be inversely proportional to their penetrance for disease. We tested this hypothesis using data from our genetic testing for medullary thyroid cancer (MTC) and pheochromocytoma/paraganglioma (PC/PGL) susceptibility genes. Frequency of each pathogenic variant found in *RET*, *SDH* subunit genes *A-D* and *VHL* in our cases was compared with its frequency in ExAC.

Genetic testing was performed in 437 Australian subjects presenting with MTC and 575 subjects presenting with PC/PGL. We identified pathogenic *RET* variants in 58 subjects (13.3%) presenting with MTC, and pathogenic *RET*, *SDHx*, or *VHL* variants in 162 subjects (28.3%) presenting with pheochromocytoma or paraganglioma. Many of these variants are reported in ExAC (for *RET* 38%, for *SDHA* 29%, for *SDHB* 20%, for *SDHC* 20% and for *VHL* 7%). None of our pathogenic *SDHD* variants are reported in ExAC. Cumulative frequency of these individually rare pathogenic variants in ExAC was 0.00015 (*RET*) and 0.0002 (*SDHA*, *SDHB*, *SDHC*), four-fold higher than expected from observed population prevalence of MTC or PC/PGL. For *RET*, the three American Thyroid Association (ATA) risk categories correlated well with groups of variants predicted to have high, moderate, or low/negligible risk of disease penetrance. For *SDHB* variants present in ExAC, our estimates predict lifetime penetrance of PC/PGL of 23% (95% confidence interval [CI] 12-39%) and lower for *SDHA* (1.5%, CI 0.5-4.2%) or *SDHC* (8%, CI 1-33%). Our findings have important implications for genetic counseling and surveillance of subjects carrying pathogenic variants in these genes.

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## Cluster I PPGLS - spanners in the Krebs cycle

**Judith Favier[1]**

*1. Inserm, Paris, France*

*SDHA, B, C,* and *D (SDHx)* genes encode the four subunits of succinate dehydrogenase (SDH), a mitochondrial enzyme of the tricarboxylic acid (TCA) cycle that oxidizes succinate into fumarate. They were the first genes encoding a mitochondrial enzyme demonstrated to act as tumor suppressors, an important finding supporting the hypothesis of a direct link between mitochondrial dysfunction and cancer proposed by Otto Warburg in the 1920's.

It is estimated that germline mutations in *SDHx* genes represent around half of inherited pheochromocytoma and paraganglioma (PPGL), which are referred to as Cluster 1 tumors. In PPGL, SDH loss-of-function results in the accumulation of succinate, which acts as an oncometabolite, by inhibiting 2-oxoglutarate-dependent dioxygenases among which HIF prolyl-hydroxylases drive a pseudohypoxic response and promote angiogenesis and DNA demethylases cause a hypermethylator phenotype.

This presentation will show how our team uses genetic and OMICs analyses on the large series of human PPGL gathered by the French COMETE network, combined with experimental studies on *Sdhb* knockout cells and xenografts to decipher these mechanisms and develop tools to evaluate the response to anti-angiogenic or demethylating therapies. It will also show how we used OMICS analyses combined with whole-exome sequencing to identify new PPGL susceptibility genes within the cluster 1 group of PPGL. Using such an approach, we previously identified the first PPGL case harboring a germline *FH* gene mutation and now discovered a new mitochondrial tumor suppressor gene encoding an unsuspected carrier. These finding demonstrate the central role of mitochondrial deficiencies in the predisposition to paragangliomas.

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## Metabologenomics of Chromaffin Cell Tumors

**Graeme Eisenhofer[1], Barbara Klink, Susan Richter, Jacques Lenders[2], Mercedes Robledo[3]**

*1. Techniche Universität Dresden, Dresden, Germany*

*2. Radboud University Nijmegen Medical Centre, Nijmegen*

*3. CNIO, Madrid , Spain*

The tremendous advances over the past two decades in both clinical genetics and biochemical testing of chromaffin cell tumors have led to new considerations about how these aspects of laboratory medicine can be integrated to improve diagnosis and management of affected patients. With germline mutations in 15 genes now identified to be responsible for over a third of all cases of pheochromocytomas and paragangliomas, these tumors are recognized to have one of the richest hereditary backgrounds among all neoplasms. Depending on the mutation, tumors show distinct differences in metabolic pathways that relate to or even directly impact clinical presentation. At the same time there has been improved understanding about how catecholamines are synthesized, stored, secreted and metabolized by chromaffin cell tumors. Although the tumors may not always secrete catecholamines it has become clear that almost all continuously produce and metabolize catecholamines. This has not only fuelled changes in laboratory medicine, but has also assisted in recognition of genotype-biochemical phenotype relationships important for diagnostics and clinical care. In particular, differences in catecholamine and energy pathway metabolomes can guide genetic testing, assist with test interpretation and provide predictions about the nature and behavior of the tumors. Conversely, results of genetic testing are important for guiding how routine biochemical testing should be employed and interpreted in surveillance programs for at risk patients. In these ways there are emerging needs for modern laboratory medicine to seamlessly integrate biochemical and genetic testing into the diagnosis and management of patients with chromaffin cell tumors.

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## Mitochondrial defects in cancer

**Darren Saunders[1], Rae-Anne Hardie[2, 3, 4], Ellen van Dam, Seher Balaban[5], Marina Pajic[2, 3], Mark Pinese[2, 3], Mary Iconomou[2, 3], Robert F Shearer[2, 3], Jessie McKenna[2], David Miller[6], Nicola Waddell[6], John V Pearson[6], Sean M Grimmond[6], Leonid Sazanov[7], Andrew V Biankin[8], Silas Villas-Boas[9], Andrew J Hoy[10, 11], Nigel Turner[1]**

*1. University of New South Wales, Sydney, NSW, Australia*

*2. Garvan Institute of Medical Research, Darlinghurst, NSW, Australia*

*3. St Vincent's Clinical School, University of New South Wales, New South Wales, Australia*

*4. Australian Institute of Health Innovation , Macquarie University, New South Wales, Australia*

*5. School of Medical Sciences and Bosch Institute, University of Sydney, Sydney, New South Wales, Australia*

*6. Centre for Medical Genomics, Institute for Molecular Bioscience, University of Queensland, Australia*

*7. Mitochondrial Biology Unit, Welcome Trust, United Kingdom*

*8. Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom*

*9. School of Biological Sciences, University of Auckland, New Zealand*

*10. Discipline of Physiology, School of Medical Sciences and Bosch Institute, University of Sydney, Australia*

*11. Boden Institute of Obesity, Nutrition, Exercise and Eating Disorders, University of Sydney, Australia*

**Background:** Pancreatic cancer has a five-year survival rate of ~8%, with characteristic molecular heterogeneity and restricted treatment options. Targeting metabolism has emerged as a potentially effective therapeutic strategy for cancers such as pancreatic cancer, which are driven by genetic alterations that are not tractable drug targets. Although somatic mitochondrial genome (mtDNA) mutations have been observed in various tumors types, understanding of metabolic genotype-phenotype relationships is limited.

**Methods:** We deployed an integrated approach combining genomics, metabolomics, and phenotypic analysis on a unique cohort of patient-derived pancreatic cancer cell lines (PDCLs). Genome analysis was performed via targeted sequencing of the mitochondrial genome (mtDNA) and nuclear genes encoding mitochondrial components and metabolic genes. Phenotypic characterization of PDCLs included measurement of cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using a Seahorse XF extracellular flux analyser, targeted metabolomics and pathway profiling, and radiolabelled glutamine tracing.

**Results:** We identified 24 somatic mutations in the mtDNA of 12 patient-derived pancreatic cancer cell lines (PDCLs). A further 18 mutations were identified in a targeted study of ~1000 nuclear genes important for mitochondrial function and metabolism. Comparison with reference datasets indicated a strong selection bias for non-synonymous mutants with predicted functional effects. Phenotypic analysis showed metabolic changes consistent with mitochondrial dysfunction, including reduced oxygen consumption and increased glycolysis. Metabolomics and radiolabeled substrate tracing indicated the initiation of reductive glutamine metabolism and lipid synthesis in tumours.

**Conclusions:** The heterogeneous genomic landscape of pancreatic tumours may converge on a common metabolic phenotype, with individual tumours adapting to increased anabolic demands via different genetic mechanisms. Targeting resulting metabolic phenotypes may be a productive therapeutic strategy.

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## Investigating the metabolism of SDH-mutated disease for novel target identification

**Daniel Tennant[1]**

*1. University of Birmingham, Birmingham, WEST MIDLANDS, United Kingdom*

Since the discovery of mutations in succinate dehydrogenase (SDH) complex early this century, it has been shown that tumours underpinned by deficiencies in this metabolic enzyme will demonstrate altered cell metabolism. However, the precise nature of these changes remains poorly described. The metabolic network within cells is highly redundant, with multiple pathways capable of synthesising the required building blocks for cell growth. By the very fact that SDH-deficient cells form tumours, we know that cells have found a way around this mutation. However, it is likely that this metabolic re-wiring has compromised their redundancy. Investigations that pinpoint the enzymes required in the SDH-deficient metabolic network may therefore lead to the identification of novel specific targets for tumours with SDH mutations.

Aspartate is a key amino acid, required for the synthesis of all nucleotides and proteins – its synthesis is therefore critical for cellular anabolism. We recently showed that cells lacking Sdhb are deficient in aspartate – a direct consequence of loss of SDH activity. SDH-deficient cells instead become reliant on the activity of pyruvate carboxylase (PC), a mitochondrial enzyme, for the majority of aspartate synthesis. We therefore showed that knockdown of PC activity results in lethality in SDH-deficient cells but has little effect on wild-type cells.

Interestingly, we have also observed perturbations in the metabolism of other amino acids, including proline and alanine, suggesting that there may be multiple opportunities for novel therapeutic targets through the investigation of this class of metabolites.

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## miR-21-3p and miR-183-5p: novel risk markers for metastatic pheochromocytoma and paraganglioma

**Bruna Calsina[1], Luís Castro-Vega[2], Lucia Inglada-Perez[1], Rafael Torres- Pérez[1], Maria Currás-Freixes[1], Rocío León[1], Cristina Montero-Conde[1], Laura Remacha Medina[1], Giuseppe Opocher[3], Henri Timmers[4], Felix Beuschlein[5], Ronald Dekrijger[6], Graeme Eisenhofer[7], Massimo Mannelli[8], Cristina Rodríguez-Antona[1], Alberto Cascon Soriano[1], Anne-Paule Gimenez-Roqueplo[2], mercedes Robledo[1]**

*1. Hereditary Endocrine Cancer Group, Human Cancer Genetics Programme, CNIO, Madrid, Spain*

*2. INSERM, Paris-Cardiovascular Research Center, Paris, France*

*3. Department of Endocrinology, Department of Medical and Surgical Sciences, University of Padova, Padova, Italy*

*4. Department of Internal Medicine, Radboud University Medical Centre, Nijmegen, Netherlands*

*5. Department of Endocrinology, University Hospital München, Munich, Germany*

*6. Department of Pathology, Reinier de Graaf Hospital, Delft, Netherlands*

*7. Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany*

*8. Department of Experimental and Clinical Biomedical Sciences Mario Serio, University of Florence and Istituto Toscano Tumori, Florence, Italy*

There is a lack of molecular markers for predicting metastases in PPGL patients, which could improve their clinical management. OMIC platforms have demonstrated to be a robust tool for identifying prognostic markers.

In this study, we used the miRNA profiling of 443 tumor samples and identified > 50 miRNA differentially expressed between benign and metastatic PPGLs. After applying strict filtering criteria, eight miRNAs were selected for validation using a well characterized independent series (n=49). Six miRNAs were validated: five upregulated (miR-21-3p, miR-182-5p, miR-96-5p, miR-551b-3p and miR-183-5p), and one downregulated (miR-202-5p).

After assessing these six validated miRNA as prognostic markers, miR-21-3p and miR-183-5p were significantly associated with a shorter time to progression. Furthermore, a stepwise logistic regression model selected these two miRNAs in combination with *SDHB* genotype as the best classifier of malignancy. Thus, we define miR-21-3p and miR-183-5p as potential novel metastatic PPGL markers.

Six remarkable proteins potentially implicated in malignant behavior of the disease were indicated from integration of the miRNA profiles and the transcriptome of these miRNA targets (Targetome) predicted *in silico*. These particular proteins highlighted the promising importance of mTOR pathway in the progression of the disease. Validation of those targets in the same independent series is being performed.

Furthermore, we have generated eight stable neuroblastoma cell lines to elucidate the phenotypic and functional implication in metastases of these miRNAs. Preliminary results show that overexpression of miR-21-3p promotes proliferation in *SDHB*-deficient and WT cell lines. We will use this model to further evaluate their implication in invasion and migration, study the validated targets *in vitro*, and we aim to identify promising metastasic specific weaknesses and strategies for the treatment of metastatic PPGL patients.

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## *In vivo* detection of succinate by magnetic resonance spectroscopy as a hallmark of *SDH*x mutations in paraganglioma

**Charlotte LUSSEY-LEPOUTRE[1], Alexandre BELLUCCI[2], Aurélie MORIN[1], Alexandre BUFFET[1], Laurence AMAR[1, 2, 3], Maxime JANIN[3], Chris OTTOLENGHI[3, 4], Franck ZINZINDOHOUE[2, 3], Gwennhael AUTRET[1], Nelly BURNICHON[1, 2, 3], Estelle ROBIDEL[1], Benjamin BANTING[2], Sébastien FONTAINE[2], Charles-André CUENOD[5, 2, 3], Paule BENIT[5], Pierre RUSTIN[1, 6], Philippe HALIMI[2, 3], Laure FOURNIER[1, 2, 3], Anne-Paule GIMENEZ-ROQUEPLO[1, 2, 3], Bertrand TAVITIAN[1, 2, 3], Judith FAVIER[1, 3]**

*1. PARIS VI University, PARIS, FRANCE*

*2. APHP, GEORGES POMPIDOU HOSPITAL, PARIS, FRANCE*

*3. PARIS V University, PARIS, FRANCE*

*4. APHP, Necker Hospital, PARIS, FRANCE*

*5. INSERM, PARIS, FRANCE*

*6. APHP, Robert Debré Hospital, PARIS, FRANCE*

**Background**

Germline mutations in genes encoding mitochondrial succinate dehydrogenase (SDH) are frequent and pejorative in patients with paragangliomas. SDH inactivation leads to a massive accumulation of succinate, acting as an oncometabolite and which levels, assessed on surgically resected tissue are a highly specific biomarker of *SDHx*-mutated tumors. The aim of this study was to detect succinate *in vivo* by magnetic resonance spectroscopy ($^1$H-MRS).

**Method**

A $^1$H-MRS sequence was optimized and applied to image nude mice grafted with *Sdhb*$^{-/-}$ (n=13) or wild-type chromaffin cells (n=16). The method was then applied in a pilot study to 9 patients with paragangliomas carrying (n=5) or not (n=4) an *SDHx* gene mutation and further validated in another 26 patients enrolled prospectively in the radiology department of the Georges Pompidou Hospital in Paris. Following surgery, succinate was measured using GC-MS/MS in resected tumors.

**Results**

In the pilot study[1], a succinate peak was observed at 2.44 ppm by $^1$H-MRS in all *Sdhb*$^{-/-}$-derived tumors in mice and in all paragangliomas of patients carrying an *SDHx* gene mutation, but neither in wild-type mouse tumors nor in patients without *SDHx* mutation. $^1$H-MRS results led to the identification of an unsuspected *SDHA* gene mutation and helped defining the pathogenicity of a variant of unknown significance in the *SDHB* gene. The ongoing validation study has already evaluated 26 patients (9 with an *SDHx* mutation, 1 VHL, 8 sporadic and 8 more patients with genetic pending results). This study revealed an estimated sensitivity and specificity of the $^1$H-MRS of 71 and 92%, respectively.

**Conclusions**

This noninvasive approach is a new, simple and robust method allowing *in vivo* detection of the major biomarker of *SDHx*-mutated tumors. The precise analysis of tumor size, location and mutation types will allow defining the limits and the power of this innovative method for routine imaging diagnosis.

1. 1 Lussey-Lepoutre et al, Clin Cancer res 2016; 22 (5): 1120

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## Succinate mediated autocrine tumorigenic effects in succinate dehydrogenase deficient paragangliomas: Potential for new targeted treatment strategies

**Dieter Matlac[1], Guang-Bo Ge[2], Luma Abunimer[3], Peter Deen[4], Henri J Timmers[5], Hendrik Lehnert[1], Zhengping Zhuang[6], Karel Pacak[3], Stephanie MJ Fliedner[1]**

*1. Neuroendocrine Oncology, Medical Department 1, University Medical Center Schleswig-Holstein, Lübeck, Germany*

*2. Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China*

*3. Section on Medical Neuroendocrinology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA*

*4. Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, Netherlands*

*5. Department of Internal Medicine, Division of Endocrinology, Radboud University Medical Center, Nijmegen, Netherlands*

*6. Neurooncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA*

Complete resection is not an option for many paragangliomas with loss-of-function mutations in succinate dehydrogenase (SDH) subunits A-D (SDHA-D, summarized as SDHx). Particularly SDHB mutations predispose to metastases, while for all SDHx mutations the prevalence of head and neck PGLs is high, often making complete resection impossible. Treatment options are thus extremely limited and prognosis is dismal once metastases are present. Identification of new therapeutic targets and candidate drugs are thus urgently needed.

Previously, we have shown that SDHx-deficient PGLs show elevated expression of succinate receptor 1 mRNA (SUCNR1). Furthermore, its ligand succinate has been shown to accumulate due to SDHx mutations. We hypothesized that autocrine stimulation of SUCNR1 plays a role in the pathogenesis of SDHx mutation-derived PGLs.

We confirmed elevated SUCNR1 protein expression in SDHx PGL tissue compared to VHL PGLs and normal adrenal medulla. To functionally investigate the influence of SUCNR1 on the viability of PGL cells, we stably transfected rat pheochromocytoma cells (PC12) with human SUCNR1 and treated them with different concentrations of succinate as well as 3 potential SUCNR1 inhibitors. Succinate treatment significantly increased proliferation of SUCNR1-transfected PC12 cells compared to control cells. The tested candidate SUCNR1 inhibitors successfully reversed this effect. In agreement with a SUCNR1 mediated effect, succinate treatment induced ERK phosphorylation.

Our data indicate that succinate has a so far unrecognized oncometabolic function in activating SUCNR1 mediated growth advances in SDHx paragangliomas. SUCNR1 thus provides a promising new druggable target for inoperable and metastatic SDHx mutation-derived tumors.

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## A gain-of-function mutation in an epigenetic modulator in patients with paraganglioma

**Laura Remacha[1], Rafael Torres-Pérez[1], Maria Currás-Freixes[1], Antonio Percesepe[2], Rocío Letón[1], Bruna Calsina[1], Susana Pedrinaci[3], Esther Korpershoek[4], Francesca Schiavi[5], Giuseppe Opocher[5], mercedes Robledo[1], Alberto Cascon Soriano[1]**

*1. Spanish National Cancer Research Centre, Madrid, MADRID, Spain*

*2. Medical Genetics - Dept. of Medicine and Surgery, University of Parma, Parma, Italy*

*3. Genetics, Hospital Universitario Virgen de las Nieves, Granada, Andalucía, Spain*

*4. Erasmus Universitair Medisch Centrum Rotterdam, Rotterdam, The Netherlands*

*5. Veneto Institute of Oncology IOV-IRCCS, Padova, Italy*

Apart from the known PPGL hereditary cases (~40%), there is still a subset of patients showing clinical features of heritability without germline mutations in the susceptibility genes identified so far. This fact strongly suggests the presence of *de novo* alterations, recessive inheritance, imprinting, or somatic mosaicism involving precursor cells.

In this study, we applied whole-exome sequencing to the germline of a parent-proband trio, a proband presenting multiple (n=7) paragangliomas and no alterations in known PPGL susceptibility genes. Genome-wide methylome analyses of mutated tissues, RNA-Seq of the trio and targeted deep sequencing of a substantial series of tumors were also performed.

Exome sequencing analysis identified a single, novel *de novo* mutation in a gene involved in epigenetic regulation, affecting a highly conserved residue located close to the aromatic cage in the protein binding site. The mutated tumors and blood tissue from the patient exhibited a unique expression and methylation profile with significant hypermethylation of several target genes (FDR<0.15), providing evidence that the mutation plays an activating role. Targeted deep sequencing revealed the presence of subclonal mutations affecting the same residue in six additional paragangliomas, all of which were head and neck PGLs and exhibited H3K9me3 positive staining. Finally, after increasing the sample set in an international collaboration effort, we were able to find an additional PGL case with a missense activating mutation affecting exactly the same protein domain and exhibiting a similar hypermethylator phenotype to the index case.

The case described herein not only further increases the number of paraganglioma susceptibility genes, but also represents, to the best of our knowledge, the first example of a gain-of-function mutation affecting a methylation gene involved in cancer predisposition.

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## Optimized procedures for diagnostic testing for pheochromocytomas/paragangliomas in patients with chronic kidney disease

**Christina Pamporaki[1], Aleksander Prejbisz[2], Frank Pistrosch[1], Mirko Peitzsch[3], Steffen Bischoff[4], Petra Mueller[4], Iris Meyer[5], Doreen Reimann[6], Andrzej Januszewicz[2], Carola Kunath[1], Jacques Lenders[7], Graeme Eisenhofer[3], Jens Passauer[1]**

*1. Department of Internal Medicine III, Uniklinikum Carl Gustav Carus at the TU Dresden, Dresden, Sachsen, Germany*

*2. Department of Hypertension, Institute of Cardiology, Warsaw, Poland*

*3. Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus at the TU Dresden, Dresden, Sachsen, Germany*

*4. Nephrologische Gemeinschaftpraxis, Dresden, Germany*

*5. Dialysis Center, Heidenau, Germany*

*6. Kidney/Hypertension/Rheumatology Center, Dresden, Germany*

*7. Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands*

Introduction:

Diagnosis of pheochromocytomas/paragangliomas in patients with chronic kidney disease (CKD) is troublesome.

Aims:

Establish optimum pre-analytical procedures for blood sampling for measurements of metanephrines and 3-methoxytyramine in patients receiving hemodialysis and optimized reference intervals for patients with stage III-IV CKD or receiving hemodialysis (HD).

Methods:

Blood was sampled before and after dialysis (including different sampling sites) in 30 patients receiving hemodialysis. Plasma concentrations of metanephrines and 3-methoxytyramine were also measured in 226 patients with CKD (79 stage III, 40 stage IV and 108 stage V) and compared to 173 subjects of an aged-matched reference population.

Results:

Among patients on hemodialysis, plasma normetanephrine concentrations were significant lower (P<0.0001) and metanephrine concentrations significantly higher (P<0.0001) in shunt than in venous blood, with no significant differences for 3-methoxytyramine. Normetanephrine, metanephrine and 3-methoxytyramine concentrations, both in shunt and venous blood, were lower at the end than before dialysis (P<0.0001). Compared with the reference population, upper cut-offs of reference intervals (97.5% percentiles) for normetanephrine, metanephrine and 3-methoxytyramine were respectively 14%, 22% and 41% higher in patients with stage III CKD and 34%, 21% and 75% higher in patients with stage IV CKD or HD. Due to the high concentrations of 3-methoxytyramine in patients with chronic kidney disease, CKD specific cut-offs were assessed only for metanephrines.

Conclusion:

These data establish optimized reference intervals for plasma metanephrines for patients with stage III-IV CKD or HD that are useful for minimizing false-positive results. In particular, using CKD-specific cut offs, it can be expected that the proportion of false positive results would fall from 7.6% to 2.5% for patients with stage III CKD and from 22.9% to 4% for stage IV CKD or HD. We also show that blood sampling in patients receiving hemodialysis is most appropriate at the end of hemodialysis and from the dialysis shunt.

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## From integrative OMICS studies to precision medicine in PPGLs

**Anne-Paule Gimenez-Roqueplo**[1, 2, 3]

*1. INSERM, Paris, France*

*2. Paris Descartes University, France, Paris*

*3. Genetics department, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, , Paris, France*

The first integrative genomic analysis of a collection of 202 PPGL, carried out by the COMETE network, demonstrated that mutation status in PPGL susceptibility genes is strongly correlated with multi-omics data and revealed the crucial role of predisposing mutations as being the main drivers of PPGL. Transcriptomic studies identified two main molecular pathways, activating either the hypoxic pathway (cluster C1) or the MAPkinase/mTOR signalling (cluster C2). DNA methylation profiling uncovered a hypermethylator phenotype specific to the tumors related to a mutation in genes encoding for a protein of the tricarboxylic cycle. Succinate acts as an oncometabolite, inhibiting 2- oxoglutarate-dependent dioxygenases, such as HIF prolyl-hydroxylases and histone/DNA demethylases, explaining pseudohypoxic signature and hypermethylator phenotype. Besides, we have recently demonstrated that metastatic phenotype is associated with subsequent immortalization mechanisms. Telomerase activation or ATRX mutations are the key somatic events linked to the metastatic progression of high-risk PPGL, especially for SDHB ones. These comprehensive analysis illustrated the functional interdependence between genomic and epigenomic dysregulations as well as highlighted the interplay between genes involved in metabolism and immortalization. Furthermore, recently published novel comprehensive multiplatform analysis of 173 PPGL led by TCGA has confirmed the COMETE data and identified recurrent fusions genes by RNA sequencing. Altogether OMICs data revealed novel diagnostic and prognostic biomarkers and new therapeutic targets for patients with metastatic PPGL. New 'omics'-based tests are going to be transferred to clinical practice to establish a precise molecular classification of primary tumor after surgery, to improve the stratification of PPGL patients at high-risk of progression, to define subgroups for targeted therapies, to establish a personalized medical management and surveillance based on circulating biomarkers. Today precision medicine is 'En Marche' and offers genuine hope in a favorable outcome in particular for patients with high-risk PPGL.

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## New genetic loci for malignant PPGLs

**Mercedes Robledo**[1]

*1. Spanish National Cancer Research Centre, Madrid, MADRID, Spain*

Extensive clinical and genetic characterization over the last 25 years has greatly improved our knowledge about the genetic basis underlying PPGLs. However, metastatic PPGL remain a diagnostic challenge, as currently there is a lack of reliable markers for stratifying high risk patients, and/or their limited predictive value continue to complicate the clinical management of PPGL patients. This is of paramount importance, and the object of intense research. Thus, application of high-throughput technologies to study PPGL has enhanced not only our understanding of tumor biology, but also has contributed to uncover potential new druggable pathways in metastatic tumours. In this regards, genome-wide DNA methylation profiling of PPGL revealed a hypermethylation phenotype, associated with mutations in Kreb's cycle related genes. This phenotype, frequently found in metastatic cases, results in the alteration of gene expression of pathways relevant for invasion and progression. On the other hand, there is a proportion of metastatic PPGLs not associated with a hypermethylation phenotype, suggesting the existence of other still unknown mechanisms. Recently, genome-wide sequencing studies have uncovered non-mutual exclusive alterations affecting different genes, as well as chromosomal fusions in some malignant cases. All this new molecular knowledge brings us closer to solving the puzzle of the biology of aggressive PPGLs.

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## Pheo-type: molecular subclassification of pheochromocytoma

**Richard Tothill**[1]

*1. Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia*

Pheochromocytomas and paragangliomas (PPGL) are remarkable not only because of their high heritability but also because of their genetic and phenotypic diversity. Up to fifteen different genes have now been linked to familial disease and additional somatic mutations in driver oncogenes have been found in sporadic tumours[1]. There is a strong association between the phenotypic properties of PPGL and underlying genetic drivers. Genotype to phenotype associations relate to clinicopathological features including cellular differentiation, excretory biochemical profile, metabolic activity, vascularisation and metastatic potential. Indeed many of these features are used to prioritise genetic testing, while the genotype of the tumour may dictate an appropriate surveillance strategy. Genome-wide molecular analysis of PPGL has revealed distinct "signatures" associated with underlying genotype of disease. Gene-expression profiling has identified between two and six subtypes[2-5]. Broadly, PPGL tumors cluster into two main subgroups – so-called Cluster 1 (C1) or Cluster 2 (C2). The C1 pseudo-hypoxia subgroup includes familial or sporadic tumors driven by germline or somatic mutations in *VHL*, *EPAS1* (HIF2A), SDH subunits *(SDHA, SDHB, SDHC, SDHD)* and other members of the Kreb cycle pathway (*FH*, *MDH2*). The C2 subgroup represents familial or sporadic tumors with underlying *RET*, *NF1*, *TMEM127*, *HRAS*, *KIF1B* and *MAX* mutations. The SDHx and VHL tumors can be further separated by distinct gene expression patterns; and C2 tumors can also be subdivided into 3-4 minor subgroups. Gene-expression profiling clearly has an important research application for improving our understanding of the biology of disease and characterising tumours that harbour new putative gene drivers by association with tumours of known genotype. It is evident that such a classification can have clinically utility, especially as an adjunct to genetic testing. We have developed a robust gene-expression assay called "Pheo-type" that enables accurate classification of PPGL tumors[6]. Pheo-type can predict the genotype of the tumour from routine diagnostic tissue. We are now using Pheo-type classification to explore other clinical and biological associations of PPGL tumours with potential imaging and therapeutic implications.

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## Structural rearrangements involving the *TERT* promoter region identified in metastatic pheochromocytomas

**Trish Dwight[1], Aidan Flynn[2], Kaushalya Amarasinghe[3], Diana E Benn[1], Richard Lupat[3], Jason Li[3], Daniel Cameron[3], Annette Hogg[3], Shiva Balachander[3], Stephen Wong[3], Bruce G Robinson[1], Sean Grimmond[4], Tony Papenfuss[3], Anthony J Gill[5], Alexander Dobrovich[6], Rodney J Hicks[7], Roderick J Clifton-Bligh[1], Richard W Tothill[7]**

*1. Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital and University of Sydney, Sydney*

*2. The Peter MacCallum Cancer Centre; and Department of Pathology, University of Melbourne, Melbourne*

*3. The Peter MacCallum Cancer Centre, Melbourne*

*4. The University of Melbourne, Melbourne*

*5. Department of Anatomical Pathology and Northern Cancer Translational Research Unit, Royal North Shore Hospital and University of Sydney, Sydney*

*6. Olivia Newton-John Cancer Research Institute, Melbourne*

*7. The Peter MacCallum Cancer Centre; and The Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne*

Pheochromocytomas and paragangliomas are neuroendocrine tumours for which the genetic drivers of metastatic progression remain largely unknown. As a result, the metastatic potential of a tumour can currently only be diagnosed by the presence of metastases. The early diagnosis of individuals at high risk of developing metastases is therefore clinically important, while the identification of any new biomarkers that are predictive of metastatic potential would be extremely valuable. Activation of *TERT* has been associated with a number of malignant tumours, including pheochromocytomas and paragangliomas. However, the mechanism of *TERT* activation in the majority of pheochromocytomas and paragangliomas remains unclear. As *TERT* promoter mutations occur rarely in these tumours, we hypothesised that other mechanisms - such as structural variations - may underlie *TERT* activation in these tumours. We observed moderate to high *TERT* expression in three of 39 tumours (35 pheochromocytomas, 4 paragangliomas), each of which were pheochromocytomas from individuals with metastatic disease and all of which lacked *TERT* promoter mutations. Using whole genome sequencing we identified, for the first time in this disease, somatic structural variants involving the proximal region of the *TERT* locus in two tumours. In both of these tumours, the structural variations led to positioning of super-enhancers proximal to the *TERT* promoter, that are likely responsible for activation of the normally tightly repressed *TERT* gene.

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## Targeted exome sequencing of Krebs cycle genes reveals candidate cancer predisposing mutations in pheochromocytomas and paragangliomas

**Alberto Cascon Soriano[1], Laura Remacha Medina[1], Susan Richter[2], Maria Currás-Freixes[1], Rafael Torres-Pérez[1], Bruna Calsina[1], Rocío León[1], Graeme Eisenhofer[2], mercedes Robledo[1]**

*1. Spanish National Cancer Research Centre, Madrid, MADRID, Spain*

*2. Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany*

The link between alterations in the tricarboxylic acid (TCA) cycle and pheochromocytoma/paraganglioma (PCC/PGL) development was first identified in 2000 when Baysal *et al* found mutations in *SDHD* in patients with the disease. Since then, mutations in seven additional genes related to the TCA cycle (*SDHA, SDHB, SDHC, SDHAF2, IDH1, FH* and *MDH2*) have been reported, which stresses the crucial role of this pathway in PCC/PGL development. Several more recent studies have identified a CpG island methylator phenotype (CIMP)-profile associated with the presence of TCA gene mutations in PCC/PGL, caused by impaired histone demethylation and 5-mC hydroxylation (5-hmC) due to the enzymatic inhibition of multiple α-KG-dependent dioxygenases. These alterations are due to the accumulation of metabolites, such as succinate, fumarate and D-2-hydroxyglutarate, in tumors carrying genetic alterations in the previously mentioned TCA cycle-related genes. In addition, tumors showing this phenotype, but no alterations in the known predisposing genes could harbor mutations in other TCA-related genes. Here we use downregulation and methylation of *RBP1,* as a marker of a hypermethylation profile, to select a series of PCCs and PGLs for targeted exome sequencing of a panel of TCA cycle-related genes. Methylation profiling, metabolite assessment and additional functional analyses were also performed in selected cases. One of the eleven tumors selected was found to carry a known cancer-predisposing somatic mutation in *IDH1.* Array methylation-based analysis uncovered a somatic epigenetic mutation in *SDHC* in a patient with multiple PCCs and GIST. Finally, two rare germline variants (one activating and the other one truncating) in new susceptibility genes were found in two tumors showing a CIMP-like profile and altered metabolites ratios. This study further attests to the relevance of the TCA cycle in the development of PCC/PGL, and points to a potential role of other metabolic enzymes involved in metabolite exchange between mitochondria and cytosol.

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## Clinical Trials for Patients with Malignant Pheochromocytomas and Paragangliomas: What Have We Learned? Where Are We Going?

**Camilo Jimenez[1]**

*1. MD Anderson, Texas, United States*

Malignant pheochromocytomas and paragangliomas are rare endocrine malignancies, for which there are no FDA-approved therapies for patients with these tumors. Until recently, patients with malignant pheochromocytomas and paragangliomas were primarily treated with surgery and chemotherapy. Surgical resection of the primary tumor is, indeed, associated with a higher survival rate and improved quality of life. Although chemotherapy is more palliative than curative, and toxicity limits its longer-term use, chemotherapy may decrease tumor burden, stabilize the disease, and improve blood pressure. Approximately, 37% of patients respond to chemotherapy.

During this century, several molecular aspects that determine the origin and development of pheochromocytomas and paragangliomas have been recognized. These aspects correlate with several of the 10 hallmarks of cancer: replicative immortality, angiogenesis, genome instability and mutations, tumor promoting inflammation, invasion and metastases, cell death resistance, sustained proliferative signaling, deregulation of energy, evasion of growth suppressors, and avoidance of immune destruction.

Several tyrosine kinase inhibitors (axitinib, cabozantinib, pazopanib, and sunitinib), two radiopharmaceutical agents (ultratrace iobenguane $I^{131}$, $Lu^{177}$-dotate) and immune therapy (pembrolizumab) are under evaluation in prospective clinical trials. These trials are exploring how these novel interventions may impact the hallmarks of cancer. We will review the rationale and mechanisms of action of these medications; analyze the results of several phase 2 clinical trials (achievement of primary and secondary endpoints and safety); and describe how these results enhance the understanding of the pathogenesis of malignant pheochromocytomas and paragangliomas.

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## Immunotherapies: a turning point for cancer treatment

**Paul Neeson[1]**

*1. Peter MacCallum Cancer Centre, Melbourne, VIC, Australia*

Over the last six years, immunotherapy has changed the treatment paradigm in a wide range of cancers. This clinical efficacy followed key findings in basic immunology that were translated to the clinic. To date, two main forms of immunotherapy have been effective, checkpoint blockade inhibitors (CBI) and chimeric antigen receptor T cells (CAR-T). Checkpoint blockade inhibitors target negative regulators of the immune response, enabling either priming of a new response and expansion of TCR repertoire (anti-CTLA4), or release of a pre-existing anti-tumour T cell response (anti-PD-1) from suppression leading to T cell activation, proliferation and effective tumour regression. A wide range of additional immunotherapy agents (eg. new CBI, agonistic antibodies, and adenosine inhibitors) are currently in clinical trial as mono- or combination therapy, indicating the initial clinical success of this form of immunotherapy may well be even more effective in the future.

CAR-T cells are T cells that have been genetically modified to express a chimeric protein encoding an antibody fragment (scFv or single chain variable fragment), transmembrane and cytoplasmic signalling domains, commonly CD28 or 41BB and CD3ζ signalling domains. Binding of the scFV to cognate antigen induces CAR-T activation, cytokine secretion, and release of effector molecules leading to tumour cell apoptosis. CAR-T cells directed to CD19 have been very successful in refractory pediatric and adult ALL, as well as refractory B cell lymphoma and CLL. In some patients refractory to CART19 therapy, subsequent CBI therapy targeting PD-1 has reinvigorated their anti-tumour response leading to systemic tumour regression. Unfortunately, this CART19 success in refractory ALL/NHL has not occurred in solid cancers. Reasons for this include issues with trafficking and persistence at the tumour site, and the immune-suppressive nature of solid tumours.

Resistance to both major forms of immunotherapy is an active area of investigation. To explore immunotherapy resistance, new technologies have been rapidly developed to enable an accurate assessment of the immune context of human tumours. This data has revealed tumour vs immune intrinsic defects contributing to resistance, as well as classifying human cancer immune context into categories. Ultimately, this information will be critical in developing algorithms to help guide patient treatment.

In conclusion, immunotherapy has now changed the treatment paradigm for many patients with cancer. The challenge for the immediate future includes increasing response rates and durability. To address this issue, clinical trials are underway exploring combination immunotherapy with therapy which targets the tumour microenvironment (eg radiotherapy, small molecule inhibitors or epigenetic modifiers) to enable penetration of the tumour by antigen-specific T cells.

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## AZEDRA® (iobenguane I 131) in patients with malignant and/or recurrent pheochromocytoma/paraganglioma (PPGL): final results of a multi-center, open-label, pivotal phase 2b study

**Daniel A. Pryma[1], Bennett B. Chin[2], Richard B. Noto[3], Joseph S. Dillon[4], Lilja Solnes[5], Jessica Jensen[6], Theresa White[6], Nancy Stambler[6], Stuart Apfel[6], Vivien Wong[6], Camilo Jimenez[7]**

*1. Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PENNSYLVANIA, United States*

*2. Duke University, Durham, NORTH CAROLINA, United States*

*3. Warren Alpert Medical School of Brown University, Providence, RHODE ISLAND, United States*

*4. University of Iowa Carver College of Medicine, Iowa City, IOWA, United States*

*5. Johns Hopkins Medicine, Baltimore, MARYLAND, United States*

*6. Progenics Pharmaceuticals, Inc., New York, NEW YORK, United States*

*7. University of Texas M. D. Anderson Cancer Center, Houston, TEXAS, United States*

Background: AZEDRA, a high specific activity, proprietary Ultratrace® form of I-131 MIBG, has been developed for the treatment of MIBG-avid metastatic and/or recurrent and/or unresectable PPGL.

Methods: MIBG-avid patients with PPGL ineligible for curative surgery, failed prior therapy or not candidates for chemotherapy, and on a stable antihypertensive regimen for tumor-related hypertension, were enrolled. 71% of subjects received at least 2 prior lines of therapy. Subjects received a dosimetric dose (111-222 MBq) followed by up to 2 therapeutic doses, each at 296 MBq/kg to a maximum of 18.5 GBq, approximately 3 months apart. The primary endpoint measured clinical benefit as defined by the proportion of patients with at least 50% reduction of all antihypertensive medications lasting ≥6 months, and the key secondary endpoint was objective tumor response (RECIST).

Results: 68 patients received at least one therapeutic dose (full analysis; FA). 50 patients received two therapeutic doses (per protocol; PP). The primary endpoint was met by 25% (95% CI 16%-37%) of FA patients, and 32% (95% CI 21%-46%) of PP patients, achieving pre-specified success criteria of the primary endpoint. 23% and 30% of evaluable FA and PP populations, respectively, achieved best confirmed tumor response of PR. 69% of FA patients and 68% of PP patients achieved best overall response of stable disease. The most common (≥50%) treatment-emergent adverse events (TEAEs) were nausea, myelosuppression and fatigue. No acute drug-related hypertensive crises were observed.

Conclusions: Clinical evidence from this study suggests that treatment with AZEDRA offers meaningful benefits to patients with MIBG-avid malignant, recurrent and/or unresectable PPGL, as measured by reduction in antihypertensive medications, and objective tumor response. AZEDRA is an effective and well tolerated treatment for an ultra-orphan disease with no approved therapies in the United States.

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## Evidence for Efficacy and Safety of Metirosine in Pheochromocytoma/paraganglioma: a Multi-center Trial in Japan

**MITSUHIDE NARUSE[1], FUMITOSHI SATOH[2], AKIYO TANABE[3], TAKAHIRO OKAMOTO[4], ATSUHIRO ICHIHARA[5], MIKA TSUIKI[6], TAKUYUKI KATABAMI[7], MASATOSHI NOMURA[8], TOMOAKI TANAKA[9], TADASHI MATSUDA[10], TSUNEO IMAI[11], MASANOBU YAMADA[12], TOMOHIRO HARADA[13], NOBUYUKI KAWATA[14], KAZUHIRO TAKEKOSHI[15]**

*1. Clinical Research Institute for Endocrinology and Metabolic Diseases, National Hospital Organization Kyoto Medical Center, KYOTO, JAPAN*

*2. Division of Nephrology, Endocrinology and Vascular Medicine, Tohoku University Hospital, MIYAGI, JAPAN*

*3. Department of Diabetes, Endocrinology and Metabolism, National Center for Global Health and Medicine, TOKYO, JAPAN*

*4. Department of Surgery II, Tokyo Women's Medical University, TOKYO, JAPAN*

*5. Department of Endocrinology and Hypertension, Tokyo Women's Medical University, TOKYO, JAPAN*

*6. Department of Endocrinology and Metabolism, National Hospital Organization Kyoto Medical Center, KYOTO, JAPAN*
*7. Department of metabolism and Endocrinology, St. Marianna University School of Medicine, Yokohama City Seibu Hospital, KANAGAWA, JAPAN*
*8. Department of Endocrine and Metabolic Diseases, Kyushu University Hospital, FUKUOKA, JAPAN*
*9. Department of Molecular Diagnosis, Chiba University Graduate School of Medicine, CHIBA, JAPAN*
*10. Department of Urology and Andrology, Kansai Medical University Hospital, OSAKA, JAPAN*
*11. National Hospital Organization, Higashinagoya National Hospital, AICHI, JAPAN*
*12. Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, GUNMA, JAPAN*
*13. Ono Pharmaceutical Co., Ltd, OSAKA, JAPAN*
*14. Ono Pharmaceutical Co., Ltd, OSAKA, JAPAN*
*15. Faculty of Medicine, University of Tsukuba, IBARAKI, JAPAN*

Metirosine is an inhibitor of catecholamine synthesis used to ameliorate catecholamine-induced symptoms of patients with pheochromocytoma/paraganglioma (PPGL), approved by the United States Food and Drug Administration in 1979. This clinical trial was conducted in accordance with the principles of the Declaration of Helsinki and the Guideline for Good Clinical Practice to receive regulatory approval of metirosine in Japan (JAPIC CTI-152999).

*Objective*:

The aim of this study was to investigate the efficacy and safety of metirosine in PPGL in a multicenter, open-label clinical trial in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice in Japan (MCAP-J Study).

*Patients*:

PPGL patients aged≥12 years requiring preoperative or chronic treatment, with baseline urinary metanephrine (uMN) or normetanephrine (uNMN) levels≥3 times the upper limit of normal values; being treated with α-blockers; and with symptoms associated with excess catecholamine.

*Results*:

Sixteen patients aged 12-86 years participated, 11 males. After 12-week treatment, the proportion of patients who achieved at least 50% reduction of uMN or uNMN from baseline as the primary endpoint was achieved in 31.3% of all patients, 23.1% under chronic treatment, and 66.7% under preoperative treatment at the last evaluation of efficacy. The changing rate for uMN and uNMN were -46.8±24.3% and -42.3±17.5%, respectively. During 24-week treatment, commonly reported adverse events (AEs) related to metirosine were sedation and somnolence in 15 patients (mild in 12, moderate in 2 and severe in 1). Serious AEs related to metirosine were sedation, anemia, and death in 1 patient each. Sedation and anemia were resolved after cessation of metirosine. The cause of death was considered by the investigator to be possibly due to the underlying unresectable pheochromocytoma.

*Conclusions*:

Metirosine was shown to be effective and tolerated in relieving symptoms by reducing excess catecholamine in PPGL patients under both preoperative and chronic treatment.

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## A storybook to help young children understand being at risk of hereditary phaeochromocytoma and paraganglioma syndrome.

**Georgina M Schlub[1], Ashely Crook[2], Jane Fleming[1], Kristine Barlow-Stewart[1], Rosie O'Shea[1], Kathy Tucker[3], Sian Greening[4]**
*1. Genetic Medicine, Sydney Medical School - Northern, University of Sydney, St Leonards, NSW, Australia*
*2. Department of Clinical Genetics, Royal North Shore Hospital, St Leonards, NSW, Australia*
*3. Hereditary Cancer Clinic, Prince of Wales Hospital, Randwick, NSW, Australia*
*4. Hereditary Cancer Clinic, Illawara Cancer Care Centre, Wollongong, NSW, Australia*

Communication with children about hereditary conditions is a challenging process for which parents often feel ill equipped. Yet, good communication strategies and psycho-education are a leading determinant of adaptation and resilience in children. In the case of hereditary phaeochromocytoma and paraganglioma syndrome (HPPS), which results in rare neuroendocrine tumors that have a high degree of genetic determinism, malignancy and an early age of onset (>5 years), genetic testing and subsequent surveillance in at-risk young children is recommended. Given the exceptionally young age at which this is done, great effort is needed to provide at-risk children and their parents with appropriate genetic counselling, support and communication strategies. Our aim has been to develop and evaluate a psycho-educational resource for young children at risk of HPPS for which no current resource exists despite the high demand. Using the practice of bibliotherapy – where stories are delivered prescriptively, we have created a storybook, for children aged 5-10 years who are at risk of HPPS. It has been developed based on what is currently known about children's developmental comprehension of heredity and disease transmission and aspires to bring them to a place of insight and comfort about what it means to be at risk of HPPS. Semi-structured qualitative interviews will be conducted and analysed thematically using a general inductive approach to ascertain the experience of ~15 parents reading this book to their children, including parents' recommendations for its improvement. We anticipate that by incorporating parents' experience, this resource, which is not only cost effective and easy to deploy, will have pyscho-educational merit that may assist parents in the process of communication with their children about HPPS.

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## Use of somatostatin analog therapy in patients with advanced pheochromocytoma/paraganglioma and somatostatin-receptor avid disease

**Vivek Narayan[1], Lauren Fishbein[2], Katherine Nathanson[1], Bonita Bennett[1], Daniel Pryma[1], Debbie Cohen[1]**
*1. University of Pennsylvania, Philadelphia, United States*
*2. Depatment of Medicine, Division of Endocrinology, Metabolism and Diabetes, University of Colorado School of Medicine , Aurora, CO, USA*

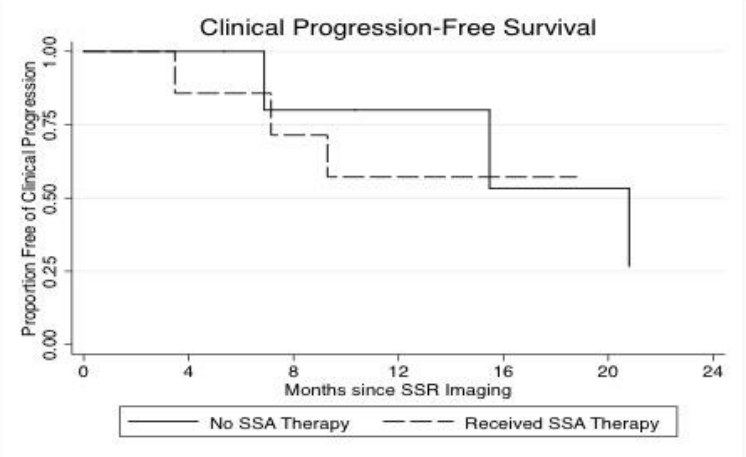
**Background:** Neuroendocrine tumors (NETs) commonly express somatostatin receptors (SSRs), as assessed by SSR scintigraphy or somatostatin analog (SSA) radiolabelled PET imaging. SSAs are used in standard management of advanced NETs and have demonstrated anti-tumor benefit in prospective trials. Pheochromocytoma/paraganglioma NETs (PPGL) may also express SSRs at levels comparable to other NETs. However, the potential benefit of SSAs in PPGL remains largely unknown, with existing literature largely limited to individual case reports. Our objective was to compare the clinical courses of a series of advanced PPGL patients with SSR avid disease treated with or without a SSA.

**Methods:** Retrospective analysis of advanced PPGL patients enrolled in a single-institution database (N = 76). Major clinical events included the initiation of systemic therapy, radiation therapy, radiopharmaceutical therapy, surgical debulking, symptomatic deterioration, or death. The Kaplan-Meier method was used to estimate clinical progression-free survival (cPFS), defined as the time from SSR imaging to the first major clinical event following the initial treatment.

**Results:** Twenty patients underwent SSR imaging (N = 14 with SSR avid disease, N = 6 with SSR non-avid disease). Of the 14 patients with SSR avidity, 8 received subsequent SSA therapy. Four of 8 patients received SSA monotherapy, while the remaining 4 received SSAs in combination with alternative systemic therapies. Seven of 14 patients developed clinical progression during study follow-up. The median cPFS was 24.7 months (IQR 7.1 – 24.7) and 20.8 months (IQR 15.5 – NR) for patients receiving and not receiving SSA therapy, respectively.

**Conclusions:** A significant portion of advanced PPGL patients demonstrate SSR avid disease and may be treated with SSA therapy, either alone or in combination with other systemic therapies. SSA therapy may provide a delay in clinical progression. This will be prospectively evaluated in a planned randomized trial.

**Figure 1. Time Until Clinical Progression, Stratified by SSA Therapy**




**log-rank p = 0.69**

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## Effective preoperative embolization of the feeding arteries just before carotid body tumor resection

**Kiyoto Shiga[1], Daisuke Saito[1], Aya Ikeda[1], Jun Miyaguchi[1], Kodai Tuchida[1], Shin-ichi Oikawa[1], Katsunori Katagiri[1]**

*1. Iwate Medical University, Morioka, Japan*

Carotid body tumor (CBT) is a rare tumor derived from carotid body paraganglion cells. It is well known that this tumor has a rich vascular network in its contents and capsules supplied by many feeding arteries. When Head and Neck Surgeons intend to resect this tumor, they sometimes experience much blood loss or injuries of carotid arteries' wall. In some cases reconstruction surgeries are required to repair or replace the damage of carotid arteries. To save blood loss during surgery of CBT, preoperative embolization of the feeding arteries is sometimes carried out. However, this procedure has been controversial about its effectiveness for the surgery so far. We planned to try embolization of the feeding arteries just before surgery and performed the surgery at the same day.

From March 2013 to September 2016, 11 patients with CBT were referred to our hospital for the radical resection of the tumor. They were 8 female and 3 male patients and their mean age was 45.4 years old ranging from 23 to 62 years old. Three patients had family history of paragangliomas. The patients underwent embolization of the feeding arteries under local anesthesia in the morning. They underwent resection of the tumor under general anesthesia just after the embolization of the feeding arteries in the afternoon of the day. The average time of surgery was 109 min. and the average blood loss was 10.5 ml. Complications of surgery in our series of patients were first bite syndrome in 3 patients, recurrent nerve palsy in 2, glossopharyngeal nerve palsy in 1, hypoglossal nerve palsy in 1 and Horner syndrome in 1.

Our " one-day procedure" resulted in obvious reduction of blood loss and operation time. We concluded that this method is superior to other procedures for the safety of CBT surgery.

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## Cardiac paraganglioma in siblings with *SDHB* mutation: Evidence for a genetic or environmental modifier?

**Amanda J Love[1], Emma Duncan[1, 2]**

*1. Royal Brisbane and Women's Hospital, Herston, QLD, Australia*

*2. Queensland University of Technology, Brisbane, QLD, Australia*

Genetic testing and surveillance of mutation carriers for hereditary paraganglioma syndromes has rapidly improved. Very poor prognosis is seen in approximately 25% of *SDHB* mutation carriers, yet low disease penetrance (25-40%) means that many *SDHB* mutation carriers undergoing surveillance will never develop disease. Current Australian surveillance guidelines (EviQ) suggest annual blood pressure and plasma metanephrines, and 2-yearly whole-body MRI. However, gadolinium toxicity is now recognised, and MRI scanning is time-consuming, costly and not widely available.

Cardiac paragangliomas are rare (<0.3%) mediastinal tumours, comprising 1-3% of primary cardiac tumours. Prior to functional imaging, most tumours were diagnosed only after onset of symptoms or signs, and thus were often large and unresectable.

We present a large kindred (68 people), of whom 28 (41%) carry an *SDHB* mutation (c.286+2T>G (IVS3+2T>G) in intron 3). Seventeen undergo annual surveillance at Royal Brisbane and Women's Hospital (RBWH). To date, six individuals in the entire pedigree have developed paraganglioma. Apart from one individual, all other cases are from one family. This family includes a carrier father, five carrier children of whom four have developed paragangliomas including two cardiac paragangliomas. None of the other SDHB mutation carriers in the family has evidenced disease, despite screening. The familial clustering of paragangliomas generally, and cardiac paraganglioma specifically, suggest the existence of a genetic or environmental modifier in this family branch.

Both cardiac paragangliomas were diagnosed on FDG-PET where no lesion was visible on whole-body MRI. Gated cardiac MRI quantified both lesions (<2cm diameter) which were successfully resected. The literature suggests that Ga-DOTATATE has excellent sensitivity and specificity for detection of SDHx-associated tumours.

Identification of a potential modifier in this family would allow for more targeted surveillance, which as a consequence of the experience described above now incorporates second yearly imaging (alternating MRI with Ga-DOTATATE) irrespective of functional assessment.

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## A case of malignant paraganglioma of urinary bladder with metastatic lesions in the bone, lung, pleura, lymph node, and esophagus

**Tatsuki Ogasawara[2, 1], Kaho Hiroshima[1], Yumiko Sasai[1], Maki Yokomoto[1], Hironobu Umakoshi[1], Mika Tsuiki[1], Tetsuya Tagami[1], Akira Shimatsu[1], Mitsuhide Naruse[1]**

*1. National Hospital Organization Kyoto Medical Center, Kyoto*

*2. Kyoto University, Kyoto, KYOTO, Japan*

Case description

A 34-year-old-man admitted to the local hospital with the complaints of headache and marked hypertension. Laboratory investigation revealed elevated levels of norepinephrine and dopamine in the plasma. Computed tomography (CT) revealed a 4×8cm tumor of the urinary bladder, while there was no evidence of metastatic disease at this stage. Pathological diagnosis of paraganglioma was obtained by surgery.

At the age of 43 years, laboratory workup showed elevated plasma dopamine and F18-FDG-PET demonstrated a recurrence of paraganglioma in the clavicle, thoracic vertebrae and rib. Uptake of 123I-MIBG was not detected in the tumors. After external radiation therapy to these sites and 4 cycles of combination chemotherapy with cyclophosphamide, vincristine and dacarbazine, the patient was followed carefully without any additional treatment. Plasma level of dopamine increased gradually and bone metastasis showed a slow progression on imaging. FDG-PET revealed a metastatic lung tumor, pleural dissemination and cervical lymph node metastasis between 46-47 years of age.

At the age of 49 years, FDG-PET demonstrated gradual growth of existing metastatic lesions, and new uptake at esophagus. Esophagogastroduodenoscopy (EGD) showed a 6mm mass at the middle intra-thoracic esophagus and biopsy was done. Hematoxylin and eosin staining of the biopsy specimen demonstrated the classic Zellballen pattern of paraganglioma with partial staining of synaptophysin and chromogranin A, suggesting metastasis of paraganglioma.

Conclusion

Little has been reported about metastasis to esophagus of paraganglioma. Differential diagnosis between esophageal carcinoid and metastasis of paraganglioma is difficult pathologically. We will report a case of paraganglioma which arised from urinary bladder initially, and was suspected to metastasize to esophagus.

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## Case: Metastatic paraganglioma with SDH C mutation

**Luke Conway[1, 2], Kathryn Berkman[1], Emily Mackenzie[1], Liza Hayes[1]**

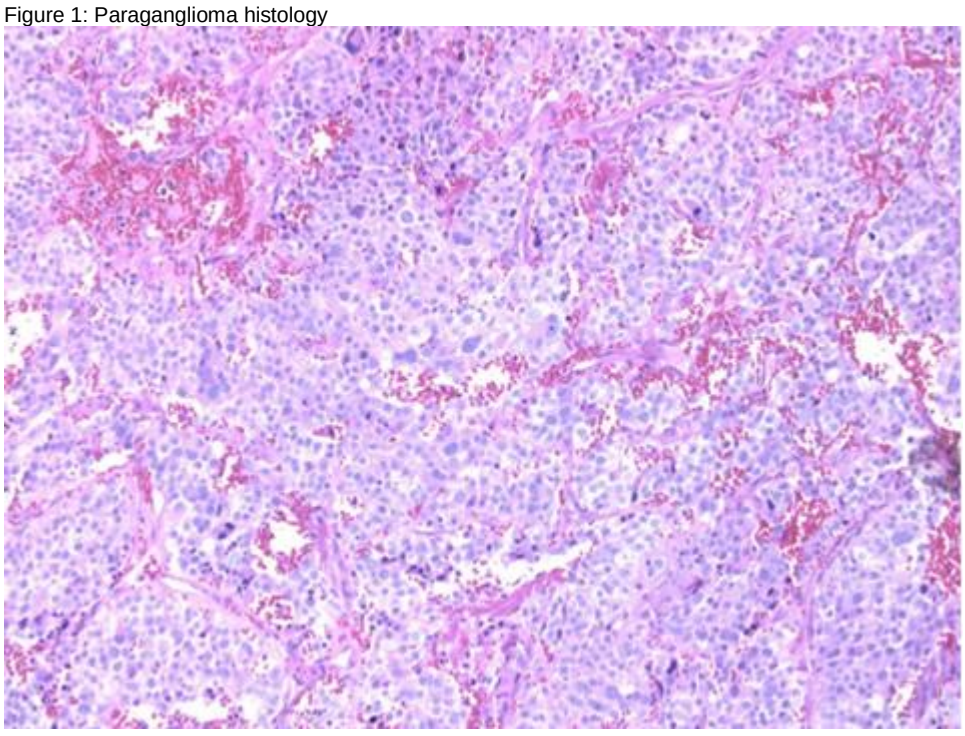
*1. Princess Alexandra Hospital, Brisbane, QLD, Australia*

*2. Mater Hospital, Brisbane, QLD, Australia*

SDHC mutations have now been described in a number of patients. These mutations are often associated with head and neck paragangliomas and are not usually malignant. We present an unusual case of a woman with an SDHC associated cervical paraganglioma with subsequent metastatic disease.

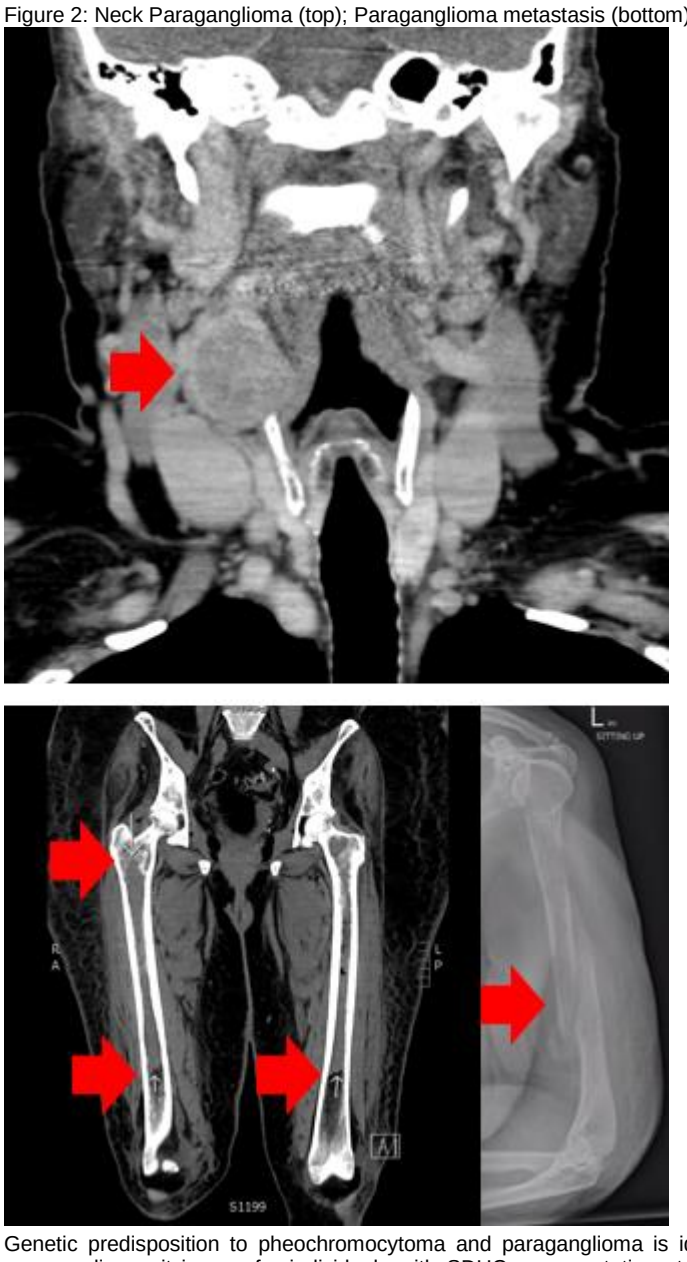
A 69 year female presented with a right neck mass. Core biopsy in 2014 confirmed a paraganglioma. Resection of the 31x31mm tumor occurred in 2016. Pre-operative 3-methoxytyramine, normetadrenaline and metadrenaline were within normal limits. Although the histology demonstrated clear margins and no evidence of malignancy in the resected lymph node, other suspicious features were present. Atypical features including large cell nests, necrosis, high ki67, increased mitosis, high cellularity and vascular invasion were identified (Figure 1). Immunohistochemistry suggested probable SDHx mutation. Next generation sequencing demonstrated a heterozygous pathogenic variant of the SDHC gene involving c.215G>A.

Figure 1: Paraganglioma histology



She presented with a left humerus pathological fracture 30 months after the initial diagnosis. Further imaging demonstrated extensive bone lesions that were confirmed on histology as paraganglioma metastasis. She had a further pathological fracture and developed multi-organ dysfunction and died within weeks of presentation.

Figure 2: Neck Paraganglioma (top); Paraganglioma metastasis (bottom)



Genetic predisposition to pheochromocytoma and paraganglioma is identified in approximately 35% of cases[1]. Whilst SDHB gene mutations are more commonly associated with malignant paraganglioma, it is rare for individuals with SDHC gene mutations to have metastatic paragangliomas[2-3]. Seven percent of head and neck paragangliomas are malignant[4]. Head and neck paraganglioma are functioning in 27% of malignant tumors, however this is a higher proportion when compared to the 5% of benign non-functioning paraganglioma[4]. Complete surgical excision is the gold standard in treatment of paraganglioma.

This case highlights the importance of active surveillance even in patients with SDHC mutations, especially in patients with atypical histology.

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## A malignant paraganglioma with intestinal fistula, anemia and SHDB gene mutation – a case report

**Zheng Pei Zeng[1], Qi Sun[2], Shi Chen[2], Lin Lu[2], Xiao Ping Xing[2]**

*1. Peking Union Medical College Hospital, 1, Shuaifuyuan Wangfujing, BEIJING, China*

*2. Endocrinology, Peking Union Medical College Hospital, Beijing*

**Objective:** To present a patient with malignant paraganglioma and intestinal fistula accompanying severe anemia by SDHB gene mutation.

**Methods:** A 29-year-old female with intestinal fistula and severe anemia was admitted again to PUMC Hospital in November 2016.

**Results:** The patient was admitted to PUMC Hospital with paroxysmal headache, palpitation and profuse sweating in 2012, and the abdominal CT scan showed a lobulated mass(3.7×3.3cm) with obscure boundary located close to the tail of pancreas. Measurement of the urinary catecholamine showed NE 318.86μg/24h(normal:16.69-40.65μg/24h), E 2.57μg/24h, DA 241.71μg/24h. $^{131}$I-MIBG image was shown as the negative but Octrotide receptor image was shown as strong positive for this mass. She was diagnosed suffer from paraganglioma. She has hypertension with Bp 188/110mmHg. After taken phenoxybenzamine therapy and the 5.0×4.0cm size of tumor was removed by laparoscopic surgery in 2013. The immunohistochemical staining shown CgA(+), Syn(+), S-100(+) and Ki-67(index 1%) in her tumor sample. Her blood pressure and urinary catecholamine level return to normal as Bp120/80, NE 28.80→14.99μg/24h, DA236.16→197.06μg/24h, E 2.88→2.14μg/24h after surgery. She presented with melena and iron-deficiency anemia(Hgb70-80g/l) at 24 weeks of pregnancy in June, 2016. CT scan showed a lobulated mass tail of pancreas with obscure boundary to intestinal tract (7.3×8.0cm), MIBG scan and octreotide scan positive. Enteroclysis revealed fistula formed between the mass and duodenal jejunal region. She had a mutation in SDHB gene.

**Conclusions:** Maignant paraganglioma is very rare disease and hard to treat. Gene testing and long-time follow-up is very important for the pheochromocytoma and paraganglioma patients.

Fig 1 The abdominal CT scan of the patient

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## PRRT for PPGLS

**Rod Hicks[1]**

*1. Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia*

Despite being rare tumours, phaeochromocytoma (PCC) and paraganglioma (PGL) have an unexpectedly diverse genomic landscape. Some genomic subtypes can be associated a high risk of metastatic disease. Whether benign or malignant, secretion of catecholamines can lead to symptoms of sympathetic over-activity and, in some cases, life-threatening acute hypertensive crisis with end-organ damage and death. This is a complex disease genotype may influence clinical behaviour and prognosis. Treatment options for metastatic or unresectable primary disease are limited with low response rates to chemotherapy and targeted therapies. $^{131}$I- MIBG has long been a palliative therapy for tumours demonstrating high uptake. Studies suggest modest effectiveness in disease stabilisation or partial responses in >50%, and improvement of blood pressure but many are unsuitable for treatment, which is also logistically challenging. Another potential novel molecular target for PCC/PGL is over-expression of somatostatin receptors (SSTR). $^{68}$Ga-labelled-peptides bind to SSTR with high affinity enabling high sensitivity imaging with PET/CT, particularly in SDHX, a subgroup with increased malignant potential. The presence of SSTR also allows theranostic application and targeting with peptide receptor radionuclide therapy (PRRT) as a novel treatment approach. There are limited published data on the outcomes of PRRT in PGL/PCC. Preliminary reports have suggested low toxicity and favourable efficacy in disease control. Our own experience suggests that this is an effective and well-tolerated option in appropriately selected patients and can be considered as a complementary treatment modality for both controlling catecholamine-related symptoms and disease progression. It is, however, a relatively complex therapy that requires an experienced multidisciplinary team. Catecholamine flare and the potential for renal disease to complicate treatment need to be anticipated and managed. Emerging options include combination of PRRT with radiosensitizing chemotherapy.

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## Removal of the primary tumor and metastases may impact the outcome of patients with malignant pheochromocytoma and paraganglioma

**Stan Sidhu[1]**

*1. Kolling, St Leonards, NSW, Australia*

Surgery is curative when a patient has a localised tumour in the adrenal gland or extra adrenal paraganglia. In the presence of metastatic disease, surgical debulking of the primary tumour to reduce catecholamine secretion intuitively seems a logical step. This talk will examine for current evidence to support or refute this concept and will discuss strategic surgical steps to minimise recurrence following adrenalectomy . There will be an emphasis on allowing the non surgeon to understand how the endocrine surgeon thinks about this disease in a multidisclipinary setting.

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## The management of Head & Neck paragangliomas – the ENT perspective

**Vincent Cousins[1]**

*1. The Alfred Hospital, Melbourne, VIC, Australia*

Paragangliomas of the head and neck HNPGL and relatively rare tumours. They may arise in the middle ear, at the base of the skull, or related to vessels and nerves in the neck. Sporadic tumours are generally solitary but up to one third of cases will have tumours that are part of a familial syndrome which may be multiple in the head and neck or associated with similar tumours in other parts of the body.

Successful management depends on comprehensive assessment including physical examination, detailed imaging, identification of those secreting hormone and genetic screening.

Management options include observation, radiation and surgery. Decision to treat and the mode of treatment chosen are strongly influenced by the patient's clinical presentation as well as specific tumour factors.

Classification systems have been developed for skull base and carotid body tumours based on imaging features of tumour site(s), spread, including the posterior cranial fossa and involvement of other structures such as the carotid artery. These are used in planning surgical approaches for resection and assessing potential morbidity of surgery. Radiation may be used as sole treatment or as an adjunct to surgery.

This presentation will cover our approach to patient assessment, treatment decision making, surgical approaches and outcomes

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## A Phase 2 Study to Evaluate the Effects of Cabozantinib in Patients with unresectable Metastatic Pheochromocytomas and Paragangliomas

**Camilo Jimenez[1], Gina Tamsen[1], Bhoomiben Patel[1], Madonna Pool[1], Roland Bassett[1], Ramona Dadu[1], Naifa Busaidy[1], Steven Waguespack[1], Mouhammed Habra[1], Aaron Jessop[1]**

*1. MD Anderson, Texas, United States*

**Background:** Malignant pheochromocytomas and paragangliomas (PPG) are frequently hypervascular and associated with bone metastases. Cabozantinib is a potent anti-angiogenic tyrosine kinase inhibitor that also targets the c-met receptor. Cabozantinib could be an effective treatment for PPG.

**Methods: I**nvestigator initiative phase 2 clinical trial with Cabozantinib to treat patients with PPG. The trial has two branches: 1. Patients with measurable disease (n=14); 2. Patients with predominant/exclusive bone metastases (n=8). Patients require histological confirmation and demonstration of disease progression over one year. Patients receive Cabozantinib at initial dose of 60 mg daily with dose reduction to 40-20 mg depending on tolerability. Primary endpoint: objective response rate (ORR); secondary endpoints: progression free-survival (PFS), blood pressure control, quality of life, and safety.

**Results:** 11 patients have been enrolled. Median age=53 years, (range 37– 78); median number of previous systemic therapy=1 (range 0 – 1). Three patients with measurable disease achieved a partial response (>30% reduction) and three patients achieved moderate responses (15-30% reduction). 4 patients with predominant bone metastases exhibited disease stabilization (as per FDG-PET) and no skeletal related events. One patient had disease progression. ORR=43%; Clinical benefit rate=91%. Tumor shrinkage has been associated with blood pressure improvement and disappearance of diabetes mellitus. Trial PFS=11.1 months (range 0.9-22.1). Toxicity has led to a reduction of the dose of Cabozantinib from a starting dose of 60 mg to 40-20 mg daily in 8 patients. Most common toxicities have been grade 1-2 fatigue, dysgeusia, and hand and foot syndrome. No grade 4 or 5 adverse events related to Cabozantinib have been reported. No molecular predictor of response has been identified.

**Conclusions:** Preliminary data shows that Cabozantinib causes tumor shrinkage in patients with measurable disease and disease stabilization in patients with bone metastases. Cabozantinib seems a safe medication for patients with PPG.

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## Long Term Follow-up in patients operated on a pheochromocytoma or a paraganglioma: a compilation of the ENS@T database.

**Laurence Amar[1], Pierre-Francois Plouin[1], Timo Deutschbein[2], Ianthe Piscaer[3], Christina Brugger[4], Laetizia Canu[5], Jerome Bertherat[6], Anthony Stell[7], Anne-Paule Gimenez-Roqueplo[8], Martin Fassnacht[2], Mercedes Robledo[9], Massimo Mannelli[5], Jacques Lenders[3], Henri Timmers[3], Felix Beuschlein[4], Olivier Steichen[10]**

*1. Hopital Europeen Georges Pompidou, Paris, PARIS, France*

*2. Department of Internal Medicine Endocrine and diabetes unit, University hopital Wuerzburg, Wuerzburg, Germany*

*3. Dept. of internal medicine, section of Endocrinology, Radboud University Medical Centre, Nijmegen, The Netherlands*

*4. Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, Munchen, Germany*
*5. Department of Experimental and Clinical Biomedical Sciences 'Mario Serio', University of Florence, Florence, Italy*
*6. Endocrinology Department, , Hôpital Cochin,, Paris, France*
*7. Melbourne eResearch Group,, University of Melbourne, Melbourne, Australia*
*8. Genetic Department, Hopital Europeen Georges Pompidou, Paris, France*
*9. Hereditary Endocrine Cancer Group, , Spanish National Cancer Research Centre, Madrid, Spain*
*10. Department of Internal Medicine, Hopital Tenon, Paris, France*
Publish consent withheld

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## Paediatric specific management challenges

**Karel Pacak[1]**
*1. NICHD, Bethesda, MD, United States*

The incidence of pheochromocytoma and paraganglioma (PPGL) in the pediatric population is estimated to be 0.2 - 0.3 cases per million children, whereas the incidence of malignant PPGLs is about 0.02 cases per million children. The recent study of Pamporaki et al. included 748 patients with PPGLs, in which 95 patients had first presentation during childhood (18 years of age or younger). Children showed statistically higher prevalence than adults of hereditary (80.4% *vs* 52.6%), extra-adrenal (66.3% *vs* 35.1%), multifocal (32.6% *vs* 13.5%), metastatic (49.5% *vs* 29.1%), and recurrent (29.5% *vs* 14.2%) PPGLs. Tumors due to cluster 1 mutations were more prevalent among children than adults (76.1% *vs* 39.3%; $P < 0.0001$) and this paralleled a higher prevalence of noradrenergic tumors in children than in adults (93.2% *vs* 57.3%). This and other studies also showed that extra-adrenal PGLs were mainly located in the abdomen. Previously, we have also showed a higher rate of *SDHB*-related metastatic and primary PPGL in children than in adults. This data suggested that all pediatric patients with PPGLs should undergo genetic testing and appropriate imaging as a guide for further treatment and follow-up. This also applies to *HIF2A*-related pediatric PPGLs, where recurrence, multiplicity, and metastasis is very common and often multiple surgeries are needed to control tumor growth and polycythemia.

Imaging algorithm for pediatric PPGL should be carefully considered due to several factors: 1. it is expected that in children with hereditary PPGLs, these tumors will recur and present as multiple/metastatic; 2. life-long follow-up is necessary; 3. radiation using CT should be limited to avoid radiation overexposure; 4. follow-up guidelines for children with hereditary PPGLs are not available. Therefore, appropriate effort must be initiated to establish such guidelines that will also include appropriate biochemical surveillance and other aspects. Recent data has suggested that at least in patients with metastatic and head and neck PPGLs, DOTA-analogs with $^{68}$Ga can be very useful and should be coupled with anatomical imaging, especially when surgery is planned. Regarding new unpublished data on pediatric patients, it is noted that $^{68}$Ga-DOTATATE PET/CT is also very useful in *SDHB*-related pediatric patients but it can be inferior to detect abdominal PGLs, where the correct detection of these tumors must be coupled with anatomical imaging and/or ultrasound. For *HIF2A*-related PPGLs in pediatric patients, we have recently shown that $^{18}$F-FDOPA is the functional imaging modality of choice.

Finally, new data on the pediatric population of patients with PPGLs showed that a prevalence of attention deficit hyperactivity disorder (ADHD) was 21% in our patients, which was significantly higher than 7.2% seen in the general pediatric population.

**Acknowledgements**
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Pamporaki et al. JCEM 2017; 102:1122.

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## Genetic approach to paediatric diagnosis

**Roderick Clifton-Bligh[1]**
*1. Royal North Shore Hospital, St Leonards, NSW, Australia*

Approximately 10-20% of all phaeochromocytomas (PCs) and paragangliomas (PGLs) occur in childhood. Paediatric PC/PGLs are likely to be associated with carriage of variants in predisposition genes: ~70% are associated with germline variants in *VHL* or *SDHB*, and less frequently in *SDHD*, *RET*, *NF1* and *MAX*. Von Hippel Lindau syndrome in childhood is associated with PC that may be bilateral, and the pathogenic *VHL* variant may be *de novo* with this presentation. Conversely, thoraco-abdominal PGLs in children are highly likely to be associated with a pathogenic *SDHB* variant; these tumours are often multifocal and/or metastatic. A striking pedigree may be observed wherein a pathogenic *SDHB* variant is diagnosed in the index child, with subsequent identification of clinically unaffected parental and grandparental carriers. Occasional reports of PGLs that exhibit loss of SDHB immunostaining without germline pathogenic *SDHx* variants suggest that epigenetic factors (such as *SDHC* promoter hypermethylation) may account for at least some of the non-hereditary component of paediatric PC/PGLs. It is however crucially important that analysis for large-scale *SDHx* deletions is performed in every paediatric PC/PGL case. Somatic mosaicism for pathogenic *EPAS1* variants defines a particular syndrome that can present in childhood: multifocal PGLs often but not always associated with congenital erythrocytosis and/or duodenal somatostatinomas.

In summary, all paediatric PC/PGL cases should be referred for comprehensive genetic testing for germline predisposition variants, and in selected cases also somatic testing for *EPAS1*. Genetic testing of families with childhood onset PC/PGL requires expert counseling and the advent of educational resources is welcome. Paediatric PC/PGL cases that are not yet diagnosed with causative genetic variants represent a unique discovery cohort for new predisposition genes.

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## Phenotype and penetrance of germline *SDHA* mutations in 30 Dutch families

**FJ Hes[1], K van der Tuin[1], AR Mensenkamp[2], CMJ Tops[1], HP Kunst[3], JC Jansen[4], EP van der Kleij-Corssmit[5], H Morreau[6], B Kusters[7], MM de Jong[8], AN van de Horst-Schrivers[9], EM Leter[10], L Spruijt[2], BMP van Nesselrooij[11], WN Dinjens[12], RA Oldenburg[13], HJ Timmers[14]**
*1. Genetics, LUMC, Leiden, ZUID-HOLLAND, Netherlands*
*2. Genetics, Radboud University Medical Centre, Nijmegen, The Netherlands*
*3. Otorhinolaryngology, Head and Neck Surgery, Radboud University Medical Centre, Nijmegene, The Netherlands*
*4. Otorhinolaryngology, LUMC, Leiden, The Netherlands*
*5. Endocrinology, LUMC, Leiden, The Netherlands*
*6. Pathology, LUMC, Leiden, The Netherlands*
*7. Pathology, Radboud University Medical Centre, Nijmegen, The Netherlands*
*8. Genetics, UMCG, Groningen, The Netherlands*
*9. Endocrinology, UMCG, Groningen, The Netherlands*
*10. Genetics, MUMC, Maastricht, The Netherlands*
*11. Genetics, UMCU, Utrecht, The Netherlands*
*12. Pathology, Erasmus MC, Rotterdam, The Netherlands*
*13. Genetics, Erasmus MC, Rotterdam, The Netherlands*
*14. Endocrinology, Radboud University Medical Centre, Nijmegen, The Netherlands*

Introduction: So far, clinical information of germline *SDHA* mutation carriers is limited to case reports and only one larger series.

Subjects and Methods: We studied 393 genetically unexplained pheochromocytoma and paraganglioma (PPGL)-patients referred for germline analysis in the Netherlands. Subsequently, clinical manifestations and disease penetrance were studied in 30 index *SDHA* mutation carriers and their 51 relatives whom were identified as carriers via cascade screening.

Results: Pathogenic germline *SDHA* variants were identified in 30 of the 393 referred PPGL-patients (7.6%). In subgroups we found mutations in: 22 of 175 patients with head and neck PGL (13%), 4 of 191 with pheochromocytoma (2%) and 4 of 27 with sympathetic PGL (15%). The mean age (± SD) at diagnosis in index *SDHA* mutation carriers was 43±16 years compared to 52±15 years in non-mutation carriers (p=0.002). The estimated penetrance of any SDHA-related disease was 12% at age 70 (95% CI 0-25%) in 51 non-index mutation carriers.

Conclusions: Pathogenic germline *SDHA* variants are frequently found in genetically unexplained PPGL patients. The majority of identified index-patients presented with an apparently sporadic PGL in head and neck. In the largest *SDHA* series assembled so far, we found the lowest penetrance of all major PPGL predisposition genes. This suggests that recommendations for genetic counselling of at risk relatives and stringency of surveillance for *SDHA* mutation carriers might need to be reassessed.

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## Systematic *in vitro* study of germline *EPAS1* variants associated with phaeochromocytoma and paraganglioma

**Edward Kim[1], Diana (Dindy) E Benn[1], Roderick J Clifton-Bligh[1], Trish Dwight[1]**
*1. Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney, NSW, Australia*

Background: Somatic variants in *endothelial PAS domain-containing protein 1 (EPAS1),* encoding hypoxia-inducible transcription factor 2-alpha (HIF2 have been reported in patients with polycythaemia and multiple paragangliomas (PGL). Our genetic testing of heritable phaeochromocytoma (PC) and PGL identified a number of germline *EPAS1* variants in subjects who also carrying pathogenic *VHL* or *SDHB* mutations. *EPAS1* variants were also identified in some PC/PGL patients without an identified germline mutation in any other known PC/PGL susceptibility gene.

Objective: Assess the functional consequences of germline *EPAS1* variants in PC/PGL.

Method: Six germline *EPAS1* variants were identified in nine PC/PGL patients: p.His194Arg, p.Arg247Ser, p.Phe374Tyr, p.Thr766Pro, p.Pro785Thr and p.Ile789Val. *In vitro* studies assessed stability of mutant HIF2 under normoxia, and effects on interaction with regulatory (VHL) and response (ARNT) proteins. GFP-tagged HIF2 expressing mutant and wild-type constructs (including

p.Pro531Thr, as a positive control) were transfected into HEK293 cells. Stability of HIF2a and effects on interacting partners were assessed by Western blot and co-immunoprecipitation (Co-IP) assays, while effects on transcriptional response and downstream target genes (*CCND1* and *SLC2a)* were assessed by luciferase reporter assay and qRT PCR.

Results: HIF2 mutants p.Arg247Ser, p.Phe374Tyr, p.Pro531Thr and p.Pro785Thr were more stable than wild-type, under normoxia. Further, significantly reduced interaction was observed between VHL and HIF2 p.Arg247Ser and p.Pro531Thr constructs ($p<0.05$), while no effects were observed between ARNT and any of the HIF2 mutants. HIF2 variants p.Phe374Tyr, p.Pro531Thr and p.Pro785Thr stimulated transcription of target genes ($p<0.05$), while p.Pro531Thr was the only variant to induce expression of both *CCND1* and *SLC2A* ($p<0.05$).

Conclusion: Germline *EPAS1* variants p.Arg247Ser and p.Phe374Tyr share some functional features in common with the known oncogenic somatic variant p.Pro531Thr. Although germline *EPAS1* variants appear to have modest effects on interacting partners and downstream targets, they may be acting as modifiers of PC/PGL.

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## Expression profiles of microRNA 183 family in pheochromocytomas

**Suja Pillai [1], Vinod Gopalan[1], Alfred Lam[1]**

*1. Griffith University, Gold Coast, QLD, Australia*

**Aim**- MicroRNA-183 cluster (miR-96/182/183) has been reported to have direct involvement in neural crest-derived tumours especially in SDHB mutated pheochromocytomas. This study aims to examine the expression profiles of miR-183 cluster in pheochromocytoma and its potential relevance to clinicopathological characteristics.

**Materials and methods** - Pheochromocytoma tissues were prospectively collected from 50 patients (28 men and 22women) who underwent resection of pheochromocytoma. MicroRNAs were extracted from these patients and converted to cDNA. MiR-183 cluster expressions were analysed by quantitative real-time polymerase chain reaction. The expression of miR-183 cluster members in pheochromocytomas was correlated with the clinical features, pathological parameters and SDHB protein expression of these patients.

**Results-** The relative expression levels of miR-183 cluster members were predominantly downregulated or deleted in pheochromocytomas. Among the cluster, low expression or deletion of miR-96 was predominantly noted in younger age patients with pheochromocytoma (<50 years, $p=0.01$). Female patients in the study group showed marked deletion of miR-182 ($p=0.05$). Deletion of the cluster was also associated with SDHB protein expression in pheochromocytomas. Moreover, patients with low miR-183 cluster expression had a slightly better survival rate when compared to patients with high expression of miR-183 cluster.

**Conclusion-** miR-183 cluster members have roles in the pathogenesis and clinical progression of pheochromocytoma. They could be potential prognostic markers in patients with pheochromocytoma.

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## Current Status of Genetic testing in Pheochromocytomas/ Paragangliomas in Japan

**Kazuhiro Takekoshi[1]**

*1. University of Tsukuba, Tsukuba, IBARAKI, Japan*

**Introduction:** Great progress has been achieved in the fields, especially genetics in pheochromocytomas/ paragangliomas . However, these findings were obtained mainly from Europe and USA. Therefore, it remains obscure whether genetic mutation plays indeed pathological role in populations outside these geographic locations. Major aim of this lecture is to overview the recent progress of genetic testing in Japan.

**Results:** 1) Since 2007, we have carried out genetic testing in 277cases in Japan. In 227 index cases, we found 81cases whose genetic mutations were

positive and summarized herein : *SDHB* 39 cases, *SDHD* 11 cases, *SDHA* 1 case,*VHL* 16 cases, *RET* 4 cases, *TMEM* 127 5 cases, *MAX* 5 cases. Consequently, notably high degree (81/227=35.7%) of patients carry genetic mutation, similar to those of previous reports from USA and Europe.

2) Growing evidence suggest that mutation of *SDHB* is highly associated with abdominal paraganglioma and the following distant metastasis.

In the present study, in 39 patients whose *SDHB* mutations were positive,

14 patients (14/39=35.9%) were developed to malignant status. Also, they mainly suffered from paraganglioma (34/39=87.2). On the contrary, analysis of blood taken from 46 cases of malignant pheochromocytomas subjects led to the identification of 14 *SDHB* mutations(14/46=30.4%). These findings regarding *SDHB* mutations are, in agreement with previous reports.

**Conclusion and perspective:** Also in Japan, 1) remarkable high degree of patients (around 40%) carry genetic mutation and 2)*SDHB* is highly involved in pathogenesis of malignant pheochromocytomas. Thus, genetic testing is prerequisite for diagnosis and treatment of pheochromocytomas/ paragangliomas in our country.

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## Catecholamine-secreting neuroendocrine tumors: Association between catecholamine and metanephrine levels and 24-hour ambulatory blood pressure monitoring before and after tumor resection.

**Jordana B Cohen[1], Anirban Ganguli[2], Lauren Fishbein[3], Daniel Pryma[1], Vivek Narayan[1], Bonita Bennett[1], Raymond R Townsend[1], Debbie L Cohen[1]**

*1. University of Pennsylvania, Philadelphia, United States*

*2. Nephrology, Georgetown University/Washington Hospital Center, Washington, DC, USA*

*3. University of Colorado School of Medicine, Denver, Aurora, USA*

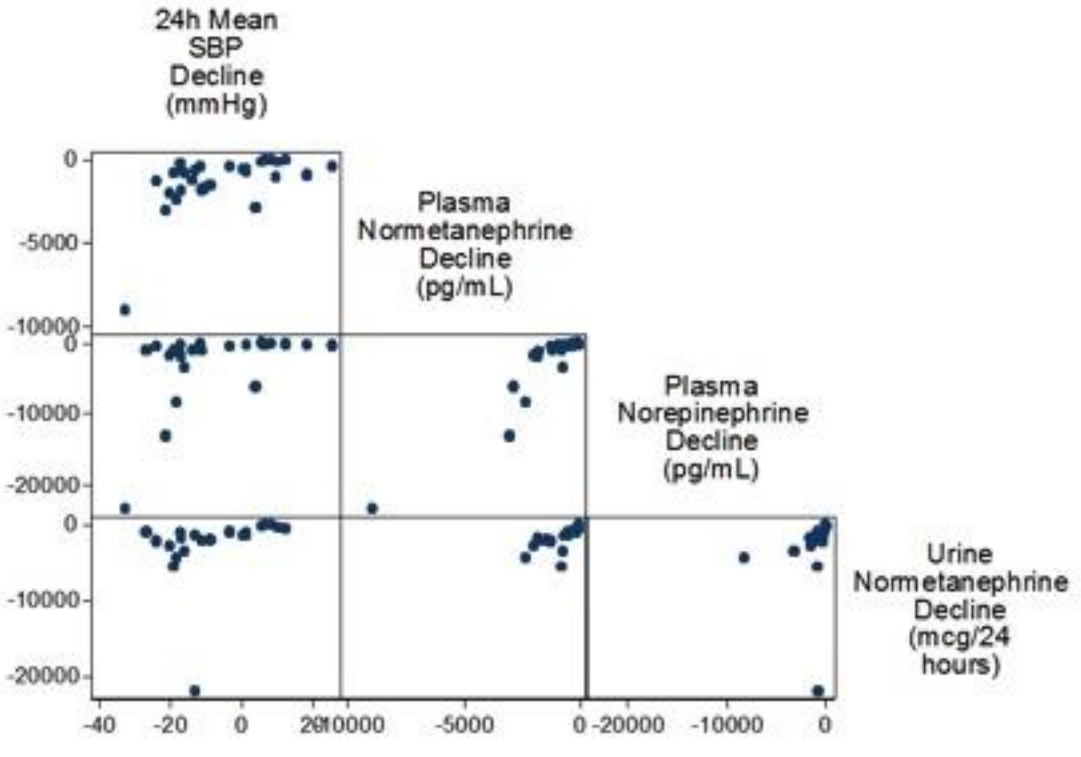
**Background:** Catecholamine and metanephrine levels usually normalize after complete surgical removal of pheochromocytomas (PCC) and paragangliomas (PGL). Little known about the relationship between decline in catecholamine and metanephrine levels and changes in systolic blood pressure (SBP) and SBP variability following tumor resection.

**Methods:** A prospective observational study of patients evaluated at the Penn Neuroendocrine Tumor Program for suspected PCC/PGL between January 2014 and December 2016. Plasma and urine catecholamine and metanephrine levels were obtained and patients underwent 24-hour ambulatory blood pressure monitoring 1-3 weeks prior to tumor resection. In patients with histologically-confirmed secretory PCC/PGL, testing was repeated 6-8 weeks post-operatively.

**Results:** 32 patients met inclusion criteria. Median age was 56 years, with 44% males (n=14), 78% Caucasians (n=25), and median body mass index 25.5 $kg/m^2$. 56% of patients (n=18) were on alpha-blockade at baseline. Compared to pre-operative values, there was a significant decline in post-operative 24-hour mean SBP (133.1 vs. 127.4 mmHg, $p=0.036$), 24-hour SBP average real variability ([ARV] 10.0 vs. 9.0, $p=0.031$), 24-hour mean pulse pressure (54.5 vs. 51.6 mmHg, $p=0.012$), and 24-hour mean heart rate (78.5 vs. 74.0 bpm, $p=0.023$). Greater decline in 24-hour SBP was associated with a greater decline in plasma normetanephrine (Spearman's rho [r]=-0.43, $p=0.017$), plasma norepinephrine (r=-0.59, $p=0.002$), and urine normetanephrine (r=-0.66, $p=0.001$). Greater decline in 24-hour SBP ARV was associated with a greater decline in plasma norepinephrine (r=-0.46, $p=0.024$). These associations were stronger among patients who were not on alpha-blockade at baseline (SBP and plasma normetanephrine r=-0.73, $p=0.005$; SBP and plasma norepinephrine r=-0.71, $p=0.015$; SBP and urine normetanephrine r=-0.76, $p=0.006$; ARV and plasma norepinephrine r=-0.71, $p=0.047$).

**Conclusion:** Decline in 24-hour SBP and SBP variability was directly associated with degree of improvement in catecholamine and metanephrine levels after PCC/PGL resection, and more evident when alpha-blockade was absent during baseline testing.

Scatterplot matrix comparing decline in 24-hour systolic blood pressure with decline in catecholamine and metanephrine levels

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## Multi-institutional study of carotid body tumors in Japan

**Aya Ikeda[1], Kiyoto Shiga[1], Daisuke Saito[1], Katsunori Katagiri[1], Koudai Tsuchida[1], Shinichi Oikawa[1], Jun Miyaguchi[1]**

*1. Iwatemedical university, Morioka, IWATE , Japan*

Carotid body tumor (CBT) is a rare disease derived from carotid body paraganglion cells. We organized a study group, the Japan Carotid Body Tumor Research Group (JCBTRG), and initiated a survey of the patients with CBT in Japan.

The study design was a multi-institutional retrospective review of medical records. Research protocols were assessed and accepted by the institutional research boards of individual institutions. A total of 316 responses were sent back to the research bureau and 150 patients were registered in our study.

From 1995 to 2015, 399 patients with CBT were referred to 112 institutions. In summary, 194 patients underwent surgery and 205 patients were under follow-up without surgery. In registered 150 cases, there were 87 female and 63 male patients and their mean and median age were 48.0 years old and 49 years old, respectively, ranging from 8 to 78 years old. Seventeen patients had family history of paragangliomas. 15 patients had bilateral CBTs. Among 93 patients who underwent surgery to remove CBT, 23 patients had tumors classified as Shamblin I, 58 as Shamblin II and 12 as Shamblin III. Mean operation time of the surgery and the mean amount of blood loss were calculated to compare.

Angiography revealed that most frequent feeding artery of these CBTs was the ascending pharyngeal artery followed by the superior thyroid artery and the occipital artery. Preoperative embolization of these arteries was effective to reduce the blood loss but operation time in Shamblin I and II tumors.

Further investigation would be needed to reveal detailed gene mutations for hereditary carotid body tumors in Japan.

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## Paraganglioma As an Independent Risk Factor for Bone Metastasis

**Maki Yokomoto-Umakoshi[1], Hironobu Umakoshi[1], Mika Tsuiki[1], Tatsuki Ogasawara[1], Kaho Hamanaka-Hiroshima[1], Yumiko Sasai[1], Tetsuya Tagami[1], Akira Shimatsu[1], Mitsuhide Naruse[1]**

*1. National Hospital Organization Kyoto Medical Center, Kyoto, Japan*

**ABSTRACT**

Context: Malignant pheochromocytoma (PHEO) and paraganglioma (PGL) (PHEO and PGL: PPGL) are frequently associated with bone metastasis. Bone metastasis requires long-term management and may lead to skeletal-related events (SREs) that remarkably reduce patients' quality of life (QOL).

Objective: The aim of this study was to elucidate the risk factors for developing bone metastasis in patients with PPGL.

Methods: The medical records of 40 consecutive adult patients with malignant PPGL at the National Hospital Organization Kyoto Medical Center between 2006 and 2016 were reviewed. SREs were defined as pathologic fracture, spinal cord compression, and the need for bone irradiation and/or surgery.

Results: PHEO and PGL were each present in 50% of the patients. Bone was the most frequent site of metastasis, detected in 60% (24/40). Bone metastasis was more frequent in patients with PGL than in patients with PHEO (*P*=0.02). A logistic regression analysis identified PGL as the only independent factor predictive of bone metastasis (odds ratio: 6.0, *P*=0.01). Half (12/24) of the patients with bone metastasis had at least one SRE. Extra-skeletal invasion of the spine, defined as local infiltration to the surrounding tissue beyond the cortical bone, was more frequently observed in patients with bone metastasis associated with SREs than without them (*P*=0.001).

Conclusions: Careful follow-up and management are warranted especially in patients with PGL as an independent risk factor for bone metastasis and in patients with extra-skeletal invasion of the spine as risk factor of SREs

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## Outcomes of long-term surveillance of succinate dehydrogenase mutation carriers followed in a familial endocrine risk management clinic

**Nirupa Sachithanandan[1, 2], Alice Hong[1], Warrick Inder[3], Mary Shannahan[2], Tess Schenberg[2], Paul James[2]**

*1. Endocrinology, St. Vincent's Health, Fitzroy, Melbourne, VIC, Australia*

*2. Peter MacCallum Cancer Centre, Melbourne, Vic, Australia*

*3. Endocrinology, Princess Alexandra Hospital, Brisbane, Qld, Australia*

Background: Asymptomatic carriers of germ line succinate dehydrogenase mutations need life-long surveillance for the development of pheochromocytomas and paragangliomas. However there is no consensus about an appropriate surveillance strategy. The aim of this study was to describe the long-term outcomes of a cohort of succinate dehydrogenase mutation carriers followed in our risk management clinic.

Method: Forty nine patients were included in the study. All patients were seen atleast once at the risk management clinic and had a minimum of one surveillance scan. Of these 12 were index cases (9 SDHB, 3 SDHD) and 37 were mutation-positive asymptomatic carriers (22 SDHB, 9 SDHD, 6 SDHC). Patients were followed for a mean of 4.4 (range 1-10) years. All patients underwent biennial MRI imaging of neck, thorax, abdomen and pelvis and annual clinic review and metanephrine testing.

Results: A total of 16 paragangliomas (10 SDHB, 6 SDHD) and 1 renal cell carcinoma (SDHB) occurred in the 12 index cases (9 SDHB, 3 SDHD). Two index patients with SDHB related paragangliomas had metastases on the initial scan. One SDHB and SDHD index patients developed additional tumours during surveillance. Among the asymptomatic carriers a total of 23 paragangliomas (22 SDHD and 1 SDHC) were detected in 8 (16%, 7 SDHD, 1 SDHC) patients. Of these 15 were detected on the first surveillance scan (14 SDHD, 1 SDHC) and 8 (all SDHD) were detected on subsequent scans. One non-index patient with SDHD mutation developed a liver metastases during surveillance. Of the seven asymptomatic carriers with SDHD mutations who had tumours on the initial surveillance scan six had the c.274G>T exon mutation in the SDHD gene.

Conclusions: Biennial MRI scans appears to be an effective surveillance strategy in the long-term follow up of patients with succinate dehydrogenase mutations, including those with SDHB.

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### Quantification of glucose metabolic rate and $^{18}$F-FDG kinetics in pheochromocytoma and paraganglioma by using dynamic PET/CT scanning

**Anouk van Berkel[1], Dennis D Vriens[2], Eric E Visser[3], Marcel M.J.R. Janssen[3], Martin M Gotthardt[3], Jacques J.W.M. Lenders[4, 1], Ad A.R.M.M. Hermus[1], Lioe-Fee L.F. Geus-Oei[2, 5], Henri H.J.L.M. Timmers[1]**

*1. Department of Internal Medicine, Division of Endocrinology, Radboud University Medical Center, Nijmegen, The Netherlands*

*2. Department of Radiology, Leiden University Medical Center, Nijmegen, The Netherlands*

*3. Department of Radiology & Nuclear Medicine, Radboud University Medical Center, Nijmegen, The Netherlands*

*4. Department of Internal Medicine III, University Hospital Carl Gustav Carus, Technical University Dresden, Dresden, Germany*

*5. MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands*

**Background:** Static single timeframe $^{18}$F-FDG PET is useful for the localization and functional characterization of primary and metastatic pheochromocytoma and paraganglioma (PPGL). $^{18}$F-FDG uptake varies between PPGL genotypes and highest standardized uptake values (SUVs) are observed in case of *succinate dehydrogenase (SDH)* mutations, probably related to enhanced aerobic glycolysis. The exact determinants of $^{18}$F-FDG accumulation remain unknown. We performed multi timeframe dynamic PET scanning to assess *in vivo* $^{18}$F-FDG kinetics to investigate whether dynamic PET has added value over static PET for distinguishing between different genotypes.

**Methods**: Dynamic $^{18}$F-FDG PET/CT was done in 26 patients with PPGL. A two-tissue compartment tracer kinetic model assuming irreversible $^{18}$F-FDG metabolism was used to estimate transfer rates of $^{18}$F-FDG between the vascular/extravascular extracellular space (EES), non-metabolized and metabolized tissue compartments. The derived transfer rates for transmembranous glucose flux ($K_1$ (in), $k_2$ (out)) and intracellular phosphorylation ($k_3$) along with the fractional blood volume ($V_b$) were analyzed using non-linear regression analysis. Glucose metabolic rate ($MR_{glc}$) was calculated using Patlak pharmacokinetic linear regression analysis.

**Results**: Both $MR_{glc}$ and maximum SUVs for cluster 1 (*SDHx,VHL*) tumors were significantly higher than those for cluster 2 (*RET, NF1)* (P<0.01) and sporadic tumors (P<0.01, P<0.05). Median $k_3$ in cluster 1 was significantly higher than for sporadic tumors (P<0.01). Median $V_b$for cluster 1 was significantly higher than for cluster 2 tumors (P<0.01). No statistical differences in $K_1$ and $k_2$ were found between the three groups. Cutoff values for $k_3$ to distinguish between cluster 1 and other tumors was established at 0.071(100% specificity, 100% sensitivity). $MR_{glc}$ significantly correlated with maximum SUV (P=0.001) and $k_3$ (P=0.002).

**Conclusion**: *In vivo* metabolic tumor profiling in patients with PPGL can be easily achieved by assessing $^{18}$F-FDG kinetics using multi timeframe dynamic PET scanning. SDH-deficient PPGLs can be reliably identified by a high $^{18}$F-FDG phosphorylation rate.

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### MIBG avidity and progression-free survival in patients with metastatic pheochromocytoma are not dependent on germline *SDHx* mutation status

**Lauren Fishbein[1], Bonita Bennett[2], Vivek Narayan[3], Katherine L Nathanson[4], Keith Cengel[5], Debbie L Cohen[2], Daniel A Pryma[6]**

*1. Department of Medicine/Division of Endocrinology, Metabolism and Diabetes, University of Colorado School of Medicine, Aurora, CO, United States*

*2. Department of Medicine/Renal Hypertension Division, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States*

*3. Department of Medicine/Division of Medical Oncology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States*

*4. Department of Medicine/Division of Translational Medicine and Human Genetics, University of Pennsylvania Perelman School of Medicine and the Abramson Cancer Center, Philadelphia, PA, United States*

*5. Department of Radiation Oncology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States*

*6. Department of Radiology/Division of Nuclear Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States*

Treatment options for patients with metastatic pheochromocytoma/paraganglioma (PCC/PGL) are limited and none are curative. Very little is known about predictors of response to systemic treatment options. Our objective was to identify predictors of response to $^{131}$I-MIBG therapy. We performed a retrospective review of 71 consecutive patients with metastatic PCC/PGL seen in a single center between January 2000 and August 2016. Fifty-five patients had $^{123}$I-MIBG scans and 45 were positive. Interestingly, there was no difference in MIBG avidity based on primary tumor location (p=0.175) or between patients with *SDHx* mutation (N=28; 26 *SDHB*, 1 *SDHD*, 1 *SDHA*) compared to those without *SDHx* mutation (N=22; 1 NF1, 21 with no mutation identified) (p=0.732). Of the 45 patients with avid disease, 51% were female (n=23) and 84% (n=38) were treated with $^{131}$I-MIBG. The mean age at treatment was 50.9 years. The mean time from initial diagnosis of PCC/PGL to metastatic disease was 5.8 years (range 0-24.5) and did not differ between those with *SDHx* mutation (n=20; 19 *SDHB*, 1 *SDHD*) and those without (n=12; 1 NF1, 11 with no mutation identified) (5.3 vs 6.3 years; p=0.683). The median clinical progression-free survival (PFS) was 34.8 months (95%CI 12.3-58.3). There was no difference in clinical PFS based on *SDHx* mutation status, primary tumor location or high vs low dose treatment (p=0.589, p=0.211, p=0.463, respectively). Limitations of this retrospective study include small sample size and lack of formal RECIST criteria. Nevertheless, these data are interesting as the results demonstrate no clinical predictors of response to MIBG therapy and do not support the notion that *SDHB* mutations carriers with metastatic PCC/PGL are less likely to be MIBG avid and have a decreased response to MIBG therapy. In summary, these data suggest that all patients with MIBG avid metastatic PCC/PGL may benefit from MIBG therapy.

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### Utility of an *in vitro* assay of SDH activity to assess functional consequences of germline *SDHB* variants

**Edward Kim[1], Venessa HM Tsang[1], Diana (Dindy) E Benn[1], Trish Dwight[1], Roderick J Clifton-Bligh[1]**

*1. Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney, NSW, Australia*

Introduction: Germline mutations in *succinate dehydrogenase subunit B (SDHB)* have been well recognised for their association with development of phaeochromocytoma (PC) and paraganglioma (PGL). SDHB immunohistochemistry, expression signature, and more recently succinate:fumarate measurement by LC/MS-MS, have been shown to functionally corroborate pathogenic *SDHB* variants. However, pathogenic validation of many *SDHB* variants has been incomplete particularly when tumour tissue is not available. We have developed (1) and refined an *in vitro* SDH assay as an adjunct for pathogenic classification of *SDHB* variants.

Objective: To assess an *in vitro* SDH activity assay for determining functional consequences of germline *SDHB* missense mutations.

Methods: Sixteen germline *SDHB* missense variants were selected for *in vitro* study. These variants encompass a spectrum of pathogenic mutations found in our genetic testing laboratory, some of which are reported in the Exome Aggregate Consortium (ExAC) and/or LOVD. HEK293 cells were transfected with either wild-type or mutant GFP-tagged SDHB constructs (generated by site-directed mutagenesis). SDH activity of complexes containing wild-type or mutant SDHB was then assessed using GFP-pulldown followed by colourimetric analysis.

Results: SDH activitys of complexes containing clinically relevant mutant SDHB were significantly lower than the wild-type control (p<0.05) with the exception of p.Ile127Ser and p.Pro197Arg. For those *SDHB* variants reported in ExAC, SDH activity was positively correlated with allelic frequency ($R^2$=0.803, p<0.05), such that more common *SDHB* variants resulted in less severe SDH dysfunction.

Conclusion: Functional assessment of *SDHB* mutation through use of SDH activity *in vitro* is a promising method for distinguishing pathogenic mutations from benign variants. Some pathogenic *SDHB* variants appear to retain SDH activity *in vitro*, and the mechanism for SDH deficiency in those cases warrants further study.

(1) Kim et al Endocrin Rel Cancer 2015;22:387-97

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### Risk factors for intraoperative hemodynamic instability for resection of pheochromocytoma and paraganglioma

**Rungsima Tinmanee[1], Sirinart Sirinvaravong[1], Thassayu Yuyen[2], Taweesak Wannachalee [1]**

*1. Division of Endocrinology and metabolism, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

*2. Department of Anesthesiology, Faculty of Medicine Siriraj hospital, Mahidol University, Bangkok, Thailand*

**Background**: Pheochromocytoma and paraganglioma (PPGL) are rare catecholamine-secreting tumors. Surgical resection of PPGL carries a risk of hemodynamic instability (HDI). Preoperative treatment with alpha blocker is known to reduce intraoperative HDI.

**Objective**: To determine predictive risk factors for intraoperative HDI in PPGL surgical resection.

**Methods**: This is a retrospective study of adult PPGL patients who underwent resection during 2003 - 2015. The patients' biochemical and imaging profiles, pre-, peri-, postoperative medical and anesthetic records were reviewed. The HDI included hypertensive, hypotensive, tachycardia, and bradycardia events.

**Results**: Seventy-four patients (64 pheochromocytoma, 10 paraganglioma) underwent 78 operations. Intraoperative HDI events occurred in 76 operations. Common HDI were hypertensive and hypotensive events (90%, 67% respectively). Rate of intraoperative vasodilator treatments were higher than vasopressor treatments (94%, 68% respectively). Significant hypertensive and hypotensive HDI ≥ 10 events, occurred in 11 operations, were associated with malignant PPGL by multivariate analysis (OR 8.1; $P < 0.01$). From subgroup analysis, tumor diameter correlated with hypertensive and tachycardia episodes (r 0.25; $P$ 0.04, r 0.75; $P < 0.01$ respectively). Anemia at presentation was associated with hypertensive HDI (OR 3.2; $P$ 0.02). Perioperative antihypertensive dose uptitration was associated with hypotensive HDI (OR 3.7; $P$ 0.04). Beta blocker usage was associated with bradycardia and postoperative hypotension (OR 1.2; $P$ 0.02, OR 2.9; $P$ 0.03). Among alpha blocker types, prazosin usage was associated with more tachycardia events than doxazosin usage (OR 8.8; $P$ <0.01).

**Conclusion**: Hemodynamic instability was common among PPGL resections, resulting in high rates of vasodilator and vasopressor usage. Malignant PPGL and larger tumors remained the common risk factors for HDI even after alpha blocker treatment. Anemia without evidence of bleeding and beta blocker usage were potentially risk factors for HDI. Types of alpha blockers may not be equally effective in preventing HDI. There was no 30-days mortality found.

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### Sporadic malignant paraganglioma with mixed biomarker expression at presentatation. Influence on management.

**Gill Vivian[1], Salvador Diaz-Cano[1], Gabriele Galata[1], Dylan Lewis[1], Ben Whitelaw[1], Klaus-Martin Schulte[1, 2]**

*1. Kings College Hospital, Camberwell, LONDON, United Kingdom*

*2. Surgery, Australian National University, Canberra, Australia*

We present 51 yr. Female Caucasian patient who, in 8/14 while on vacation became aware that her clothes became tight. She was well, had consulted a Cardiologist 12 months previously with an episode of palpitations (assumed SVT), which did not recur with reduced caffeine.

She had no signs of cortisol or androgen excess. Abdominal examination, Imaging and Biochemistry LUQ 24x20x17 cm complex retroperitoneal vascular mass separate to normal solid abdominal organs with pulmonary nodules and elevated Plasma and urinary catecholamines. Imaging biomarkers showed a mixed pattern of MIBG, SSR (Dotatate) and FDG avidity in primary, lung, liver and bone metastases. Oncological resection of 26 cm tumour with extra-adrenal invasion, Ki67>3%, PASS 20/20. Genetic screening was negative for all mutations.

She remained well in full employment during 12 mo therapy with 4 x I131 MIBG (35 GBq) and Lu177 Dotatate (7.4 GBq) as normal marrow. The metanephrines normalized with Recist response to liver, lung and some bone metastases; a large lytic painful scapular metastasis was treated with radio- and cryo-therapy.

Sunitanib was declined for residual FDG positive small lung metastases due to her concerns over benefit and side effects; she remained clinically stable and continued to work.

At 20 mo. she contracted H1N1 influenza on vacation, became short of breath and at CTPA multiple large pulmonary metastases and mediastinal nodes. In absence of clinical trials Immunomodulating therapy was not offered, as the tumour was PDL1, PD1 negative.

She proceeded to palliative care and died 24 mo. after presentation.

This patient demonstrates the increasing importance of integrating all histological, biochemical and imaging biomarkers in therapy choice to optimize patient quality of life during survival. We postulate the precipitation of relapse by viral infection with possible immune suppression.

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## Genetic Aetiology of Paediatric Phaeochromocytoma and Paraganglioma: Experience of a Tertiary Centre and Review of Literature

**Anand Vasudevan[1], Katherine Tucker[1], Roderick Clifton-Blight[2, 3], Diana Benn[2, 3]**

*1. Prince of Wales Hospital, Randwick, NSW, Australia*

*2. Cancer Genetics, Kolling Institute, Royal North Hospital, Sydney, NSW, Australia*

*3. University of Sydney, Sydney, NSW*

Phaeochromocytomas (PC) and paragangliomas (PGL) occurring in the paediatric age group are highly likely to be associated with genetic predisposition. Common genes associated with paediatric PC/PGLs are *VHL* (in Von-Hippel-Lindau syndrome), *SDHx* (hereditary paraganglioma syndromes 1-5), *RET* (Multiple endocrine neoplasia-2) and *NF1*.

A clinical audit of 13 paediatric PC/PGL patients (11 unrelated and two siblings who presented independently) seen through our clinic between 1993 and 2017 was performed. Genetic testing was performed according to a clinical algorithm (1): Iterative testing of *VHL*, *RET*, *SDHB*, *SDHD*, *SDHC* and *SDHA* by Sanger sequencing (and MLPA in selected cases) was performed according to a clinical algorithm (1); *MAX* and *TMEM*127 were sequenced in two cases. A germline molecular cause was identified in nine (69%): six had pathogenic variants in *SDHB* and the three had pathogenic variants in *VHL* . Five of six patients with an *SDHB* mutation presented with extra-adrenal tumours. All *VHL* carriers and one *SDHB* carrier presented with PC.

In four patients without a pathogenic germline variant, none had phenotypic features to suggest neurofibromatosis-1 or somatic *EPAS1* mutation. Strikingly, one case demonstrated loss of SDHB IHC despite absence of *SDHx* pathogenic variants, including assessment for large deletions by MLPA. It is possible that this represents another case of somatic epigenetic inactivation of *SDHC* (2), and confirmatory methylation studies are pending. Next-generation panel sequencing of all four cases is also pending.

In conclusion, paediatric PPGLs are highly likely to be associated with pathogenic germline variants in *VHL* or *SDHx* (particularly *SDHB*). This high a priori likelihood of PC/PGL genetic predisposition has two important corollaries: paediatric PPGLs should routinely have comprehensive genetic testing for all predisposition genes (including somatic testing for *EPAS1*); and new modes of genetic predisposition are more likely to be discovered in “negative” cases with younger onset of disease.

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## tsAM5NE cells: a novel mouse cell model for pheochromocytomas and paragangliomas?

**Peter MT Deen[1], Marjolein C ter Laak[1], Selma Waaijers[1, 2], Henri JLM Timmers[2]**

*1. Physiology, Radboud Univ. Med. Center, Nijmegen, Netherlands*

*2. Laboratory Medicine, Radboud Univ. Nijmegen Med Center, Nijmegen, Gelderland, The Netherlands*

**Introduction:** Pheochromocytomas and paragangliomas (PPGLs) are neuroendocrine tumours originating from chromaffin tissue of the adrenal medulla or from extra-adrenal sympathetic and parasympathetic paraganglia. Approximately 40% of all cases are caused by a germline mutation. PPGLs due to mutations in the β-subunit of the succinate dehydrogenase (SDHB) have the highest metastatic rate, for which effective systemic therapy is lacking. To better understand the pathophysiology and for identification and testing of therapeutic targets, a proper cell model is warranted. We therefore analysed the potential of a temperature-inducible-differentiating murine adrenal medullar cell line, tsAM5NE cells.

**Methods:** tsAM5NE cells are derived from the adrenal medulla of mice transgenic for the thermo-sensitive T-antigen, which proliferate at 33ºC and, due to inactivation of the T-antigen, differentiate to adrenal medulla cells when grown at 39ºC. Catecholamine storage was determined of cells stimulated with and without the glucocorticoid hormone dexamethasone for three days, grown at 33ºC and 39ºC.

**Results:** <5% of the tsAM5NE cells survived 3 days at 39ºC, whereas all cells survived 3 days at 33ºC. When grown at 33ºC, noradrenaline and adrenaline content was 246.8±6.0 nmol/$10^9$ cells and 0.1±0.04 nmol/$10^9$ cells, respectively. Adrenaline (7.5±0.5 nmol/$10^9$ cells), but not the noradrenalin (210.3±10.0 nmol/$10^9$ cells), content was markedly increased following dexamethasone stimulation. At 39ºC, noradrenalin and adrenalin levels were 49.25±3.9 and <0.9 nmol/$10^9$ cells, respectively, which increased to 137.1±24.34 and 89.9±15.4 nmol/$10^9$ cells with dexamethasone, respectively. The cells showed an increased adrenaline/noradrenaline ratio upon dexamethasone stimulation when grown at 39ºC (0.656) compared to 33ºC (0.036) indicating a more differentiated phenotype when growing at 39 ºC.

**Conclusion:** tsAM5NE cells closely replicate the dexamethasone sensitive secretory phenotype of chromaffin cells and hold promise for the development of a model system for SDHB deficient PPGL.

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## The microenvironment induces collective migration in *SDHB* silenced mouse pheochromocytoma spheroids

**Elena Rapizzi[1], Vanessa D'Antongiovanni[1], Serena Martinelli[1], Susan Richter[2], Letizia Canu[1], Daniele Guasti[1], Paolo Romagnoli[1], Karel Pacak[3], Graeme Eisenhofer[2], Massimo Mannelli[1]**

*1. University of Florence, Florence, ITALY, Italy*

*2. University Hospital Carl Gustav Carus, Dresden, Germany*

*3. National Institutes of Health, Bethesda, USA*

We evaluated the effects of *SDHB* silencing in a three dimension (3D) culture using tumor spheroids of a mouse pheochromocytoma cell line silenced or not (wild type = wt) for the catalytic SDHB subunit. Moreover, by co-culturing *SDHB* silenced or wt spheroids with primary cancer-activated fibroblasts (CAFs), we investigated the role played by the microenvironment on spheroid growth and migration/invasion processes.

Spheroid growth was rapid and similar in both wt and *SDHB* silenced spheroids. When spheroids were conditioned by CAFs medium, both wt and *SDHB* silenced spheroid diameter was significantly smaller, but the cloud of cells surrounding *SDHB* silenced spheroids was wider. Indeed, when spheroids were co-cultured with fibroblasts, *SDHB* silenced cells showed a significant increase in matrigel invasion as demonstrated by the computation of the migratory areas ($p<0.001$). Moreover, cells detaching from the *SDHB* silenced spheroids moved collectively, unlike the cells of wt spheroids that moved individually. Additionally, *SDHB* silenced spheroids developed long filamentous formations along which clusters of cells migrated far away from the spheroid, while these structures were not present in wt spheroids. Looking for environmental factors responsible for these effects, we added lactate, that we found to be largely secreted by CAFs, to the culture medium and we found that lactate plays a specific role in promoting migration only of *SDHB* silenced cells.

In this work, we demonstrated that *SDHB* silencing *per se* increases tumor cell migration/invasion and that microenvironment, as represented by CAFs, plays a pivotal role in enhancing collective migration/invasion in Pheo *SDHB* silenced tumor cells, suggesting their role in increasing the tumor metastasizing potential.

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## Expanding the phenotype of the fumarate hydratase germline mutation familial cancer syndrome

**Veronica Boyle[1], Kelly Sullivan[1], Trish Dwight[2], Diana (Dindy) Benn[2], Anthony Gill[2], Roderick Clifton-Bligh[2], Michael Croxson[1]**

*1. Auckland District Health Board, AUCKLAND, New Zealand*

*2. Kolling Institute of Medical Research, The University of Sydney, Sydney, New South Wales, Australia*

Germline pathogenic variants in the Krebs cycle enzyme *Fumarate hydratase* (FH) have been associated with Hereditary Leiomyomatosis and Renal Cell Cancer syndrome (HLRCC) and pheochromocytoma. We describe a kindred in whom phaeochromocytoma (PC) and/or paraganglioma (PGL) was associated with a pathogenic variant c.1142C>T (p.Thr381Ile) mutation in exon 8 of *FH*. We also describe, for the first time, the association of *FH* with gastrointestinal stromal tumour (GIST) in one affected case.

The proband presented aged 31 years with hypertension and was found to have a phaeochromocytoma, which was resected. He had a non-secretory paraaortic paraganglioma aged 54 years. Other family members affected by PC/PGL include a niece and two cousins. The proband provided consent for genetic testing and his DNA was sequenced on a targeted amplicon panel, which discovered the heterozygous *FH* pathogenic variant. Immunohistochemistry of his paraganglioma confirmed FH deficiency. The variant has been identified in other family members.

The proband's affected mother (aged 78 years) was recently treated for GIST, discovered during investigation of syncope and hypotension. CT abdomen showed a 7.7cm x 7.2cm x 7.1cm mass, appearing to arise from the left adrenal gland. On the basis of mildly raised plasma normetanephrine (1839 and 1338pmol/L, NR <900pmol/L), the mass was suspected to be a phaeochromocytoma and the patient was treated with phenoxybenzamine prior to surgery. It was apparent intraoperatively that the mass arose from the greater curvature of the stomach. A partial gastrectomy was performed and histology confirmed a GIST. Immunohistochemistry staining was CD117 (KIT), CD34 and DOG1 positive. FH staining showed a normal pattern with distribution within the cytoplasm.

To our knowledge this is the first report of a GIST in a person with an FH germline mutation. In this patient, it is uncertain whether the GIST was sporadic or represents a previously unrecognized FH phenotype.


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## Utility of chromogranin A as a predictive marker for paraganglioma surveillance in succinate dehydrogenase B and D


**Michael JW Thompson[1], Venkat Parameswaran[2], John R Burgess[2, 3]**

*1. Department of General Medicine, Royal Hobart Hospital*

*2. Department of Diabetes and Endocrinology, Royal Hobart Hospital*

*3. School of Medicine, University of Tasmania*



Background:

Succinate dehydrogenase B (SDHB) and D (SDHD) mutations are associated with increased risk of paraganglioma. The approach for detecting incident disease continues to be refined with contemporary algorithms emphasising specialised imaging and biochemical studies. We examined the utility of chromogranin A (CgA) to predict composite paraganglioma surveillance outcome in patients with SDHB and SDHD mutation.

Methods:

A retrospective audit of patients (*n*=25, 40% female) with SDHB (*n*=22) or SDHD (*n*=3) mutation who underwent composite surveillance for paraganglioma at our institution between 1 July 2011 and 30 May 2015. All patients had contemporary (within six months) measurement of CgA concentration. Composite surveillance for paraganglioma included measurement of urinary catecholamines, plasma metanephrines and 18F-FDG PET/CT scanning. Patients were considered positive for an abnormality if any one of the composite tests were positive.

Results:

There were seven positive composite results during the study period, four of whom had elevated CgA concentrations. CgA had a sensitivity of 57% and specificity of 78% for predicting a positive composite result. The positive and negative predictive value of raised or normal CgA concentrations were 50% and 82%, respectively. SDHD mutation carriers contributed to two of three false negative results. In patients with SDHB mutation the sensitivity and the negative predictive value of CgA were 80% and 93%, respectively.

Conclusion:

CgA is a useful component of SDHB surveillance, with a normal result indicative of negative findings on 18F-FDG PET/CT and catecholamine screening.


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## Pheochromocytoma in Pregnancy: Case Series from a Tertiary Center from Thailand


**Parichat Yimnoi[1]**

*1. Medicine, Excellence center of Diabetes, Hormones and Metabolism, King Chulalongkorn Memorial Hospital The Thai Red Cross Society,Chulalongkorn University, Pathumwan, BANGKOK, Thailand*


Publish consent withheld

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## Coexistence of Hepatic Metastasis from Malignant Pheochromocytoma and Hepatocellular carcinoma, A Case Report

**Natnicha Houngngam**

**Coexistence of Hepatic Metastasis from Malignant Pheochromocytoma and Hepatocellular carcinoma, A Case Report**

Natnicha Houngngam, M.Sc., Parichat Yimnoi, M.Sc., Thiti Snabboon, M.D.

Excellence Center of Diabetes, Hormone and Metabolism,Division of Endocrinology and Metabolism, Department of Medicine,Faculty of Medicine,Chulalongkorn University,King Chulalongkorn Memorial Hospital,Bangkok,Thailand

Background: Some distinct features of imaging study may raise the diagnosis of pheochromocytoma;however, several imaging characteristics can be overlapping with those seen in other hypervascular tumors.

Aim:We report an unusual presentation of malignant pheochromocytoma with liver metastasis coexisting with hepatocellular carcinoma (HCC).Proposed imaging characteristics to differentiate between these tumors were discussed.

Case presentation:A 42-year-old previously healthy man complained about upper abdominal pain for 1 week.Physical examinations were unremarkable except for a non-tender large abdominal mass at his left upper quadrant.Computerized tomography demonstrated a 5-cm liver mass accompanied with an 11-cm left suprarenal mass. The diagnosis of HCC was confirmed with liver biopsy and an elevated a-fetoprotein level. He is also seropositive for hepatitis B infection.Hepatic wedge resection with left adrenalectomy for presumed adrenal metastasis was performed,with a noticeable finding of abruptly rising in blood pressure up to 200/120 mmHg during manipulation of the adrenal mass. Pathological reports of the adrenal mass and the liver nodules of the segment IV and II from the wedge resection were compatible with pheochromocytoma. Additional findings from 131-Iodine-metaiodobenzyguanidine ($^{131}$I-MIBG) scintigraphy and hormonal studies confirmed the coexisting tumors of HCC and malignant pheochromocytoma with liver metastasis. Serial courses of TACE for treatment of HCC and malignant pheochromocytoma and systemic chemotherapy were given with initial clinical response. Unfortunately,the patient has died from hepatic failure at the 18-month follow-up period.

Conclusion:We report an unusual case of co-existence of liver masses from HCC and malignant pheochromocytoma.The clinicians should be aware of this condition to prevent catastrophic event from pheochromocytoma crisis,especially in the endemic areas of HCC.

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## Anti-tumorigenic and anti-metastatic activity of Aeroplysinin-1, a sponge-derived marine drug on mouse pheochromocytoma cells

**Nicole Bechmann[1], Hermann Ehrlich[2], Graeme Eisenhofer[1], Stefan R. Bornstein[3], Christian G. Ziegler[3]**

*1. Institute of Clinical Chemistry and Laboratory Medicine , Technische Universität Dresden, Dresden, Germany*

*2. Institute of Experimental Physics, TU Bergakademie Freiberg, Freiberg, Germany*

*3. Department of Medicine III, Technische Universität Dresden, Dresden, Germany*

Pheochromocytomas and paraganliomas (PPGLs) are neural crest-derived tumors with variable aggressiveness. Over 10% of PPGLs patients have malignant disease at their first surgery with a 5 year survival of >50% (Eisenhofer, et al. 2004). The development of malignancy and the underlying molecular pathways in PPGLs are poorly understood and efficient treatment strategies are missing. Marine sponges provide a natural source of promising anti-tumorigenic and anti-metastatic agents. The present study evaluates the anti-tumorigenic and anti-metastatic potential of Aeroplysinin-1 (García-Vilas, et al. 2015), a secondary metabolite isolated from the marine sponge *Aplysina aerophoba,* on mouse pheochromocytoma cells (MTT).

Aeroplysinin-1 decreased cell viability ($EC_{50}$ = 11.4 µM) and induced apoptosis in a concentration dependent manner. Moreover, treatment with Aerolysinin-1 diminished the number of proliferating cells (*2D*-culture) and reduced spheroid growth (*3D*-culture) significantly. The influence of Aeroplysinin-1 on pro-metastatic behavior was analyzed using Boyden Chamber assays. Aeroplysinin-1 decreased the migration ability of the cells significantly (P = 0.01), whereas, the invasion capacity was not affected. MTT cells showed a low adhesion affinity to the extracellular matrix protein fibronectin that is mildly affected by treatment with Aeroplysinin-1. In contrast, Aeroplysinin-1 significantly diminished the high adhesion capacity of the MTT cells to collagen (P < 0.001) and, furthermore, reduced the ability to form spheroids significantly. Ongoing investigations indicate a regulative influence of Aeroplysinin-1 on the expression of the different integrins and cadherins, potentially explaining the inhibitory effects of Aeroplysinin-1 on pro-metastatic behavior.

These *in vitro* investigations show promise for the application of the sponge-derived marine drug, Aeroplysinin-1 as anti-tumorigenic and anti-metastatic drugs against PPGLs. Furthermore, screening of other sponge-derived secondary metabolites provides an auspicious strategy to identify novel therapeutic strategies for metastatic PPGLs.

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## Metabolomic analysis identifies novel tumour-causing mutations in fumarate hydratase in two patients with phaeochromocytoma

**Susan Richter[1], Bruna Viana[2], Thanh Huynh[2], Jacques Lenders[3], Mercedes Robledo[4], Graeme Eisenhofer[1], Karel Pacak[2]**

*1. Department of Clinical Chemistry and Laboratory Medicine, Uniklinikum Dresden, The Dresden University of Technology, Dresden, Germany*

*2. Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, USA*

*3. Department of Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands*

*4. Hereditary Endocrine Cancer Group, CNIO, Madrid, Spain*

Germline fumarate hydratase mutations (*FH*) are a well-known cause of renal cell carcinoma and leiomyoma, but were only described in 2013 to also drive phaeochromocytoma/paraganglioma (PPGL) development. So far nine individuals with PPGLs due to familial *FH* mutations have been described. Here we add to the list by presenting the cases of two women with *FH* mutations previously not reported in PPGL.

Metabolic screening of Krebs cycle metabolites by LC-MS/MS identified two phaeochromocytomas with fumarate of 500 and 114 ng/mg tissue (median of 421 samples 1.87 ng/mg, 97.5 percentile 10.425 ng/mg, 2.5 percentile 0.085 ng/mg). Next generation panel sequencing, validated by Sanger sequencing, identified a novel disease-causing variant in the *FH* gene in Patient 1 (c.700A>G p.Thr234Ala) and a variant only reported in a paediatric case of FH deficiency in Patient 2 (c.T908C p.Leu303Ser). Both missense mutations are described as damaging with Polyphen-2 scores of 0.632 and 1.0, respectively. Loss-of-heterozygosity was confirmed in the tumour tissue.

Both patients presented with hypertension and symptoms of catecholamine excess, and after referral to the specialist centre (2006; 2011) right adrenal masses were detected. In both cases, biochemical workup showed urinary and/ or plasma increases confined to normetanephrine. Patient 2's father suffered from renal cell carcinoma; whereas Patient 1's family history presented with a thyroid tumour and melanoma. Until now there is no clear indication of metastatic disease, but follow-up is continuing.

Considering all *FH*-mutated PPGL cases to date, germline *FH* mutations appear to predominantly lead to adrenal phaeochromocytomas (9 of 11 cases) with a noradrenergic biochemical phenotype. As reported earlier, patients are at risk of multiple tumours, recurrence and metastatic disease emphasising the need for identification of these patients and regular follow-up.

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## Deciphering genetic aberrations through metabolic profiling: 2-Hydroxyglutarate elevations in phaeochromocytoma/paraganglioma

**Susan Richter[1], Laura Gieldon[2], Anastasios Mangelis[1], Mirko Peitzsch[1], Felix Beuschlein[3], Henri Timmers[4], Massimo Mannelli[5], Karel Pacak[6], Daniela Aust[7], Alberto Cascon[8], Mercedes Robledo[8], Klink Barbara[2], Eisenhofer Graeme[1]**

*1. Department of Clinical Chemistry and Laboratory Medicine, Uniklinikum Dresden, The Dresden University of Technology, Dresden, Germany*

*2. Institute for Clinical Genetics, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany*

*3. Medizinische Klinik and Poliklinik IV, Ludwig-Maximilians-Universität München, München, Germany*

*4. Department of Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands*

*5. Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence and Istituto Toscano Tumori, Florence, Italy*

*6. Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, USA*

*7. Institute of Pathology and UCC Tumor and Normal tissue bank, Uniklinikum Dresden, The Dresden University of Technology, Dresden, Deutschland*

*8. Hereditary Endocrine Cancer Group, CNIO, Madrid, Spain*

D- and L-2-hydroxyglutarate (D/L-2HG) are metabolites normally present in low concentrations. Elevations of D-2HG are attributed to gain-of-function mutations in isocitrate dehydrogenase 1 or 2 leading to excessive formation of D-2HG from α-ketoglutarate (αKG). Other reports identified that promiscuous activity of lactate dehydrogenase-A, malate dehydrogenases and phosphoglycerate dehydrogenase contribute to L-2HG production. Both enantiomers are able to inhibit αKG-dependent enzymes leading to pseudohypoxia and changes in genome methylation through effects on prolyl hydroxylases and histone/DNA demethylases. In phaeochromocytoma/paraganglioma (PPGL) only two patients with somatic *IDH1* mutations have been described so far.

This study investigated the frequency of abnormal tissue formation of both 2HG enantiomers in PPGL. We established a database of central carbon metabolites for 397 PPGLs using LC-MS/MS. Outlier analysis of normalised 2HG values resulted in the top 31 samples being marked as outliers. These 31 PPGLs had various mutational backgrounds. Nine PPGLs with the highest 2HG levels were investigated in more detail (5 of these had unknown mutational status). Four of nine PPGLs showed not only high 2HG but also high αKG (#1,3,5,6). Tumour #1 (*IDH1*-mutation) and #2 had strong elevations of D-2HG relative to L-2HG (127- and 901-times, respectively), confirming the functionality of the *IDH1* mutation. Tumour #3 and #4 showed 13-times higher L-2HG. In PPGL #3 the highest levels of glutamine were measured. Tumour #5 and #6 had the highest αKG to citrate ratios of the set.

Genetic testing by next generation sequencing using a panel of known cancer-associated genes and Krebs cycle related genes was used to relate identified metabolic profiles to genetic aberrations. Consistent with high D-2HG levels, tumour #2 carried an *IDH2* mutation (c.514A>G, p.Arg172Gly). To our knowledge, the first one reported in PPGL.

Inexpensive metabolite profiling is a valid tool to guide mutation testing and screen for variants with potentially unknown significance.

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## Survey of RCPAQAP participants regarding their measurement and reporting of plasma metanephrines

**Wilson Punyalack[1], Gerald Woollard[1], Malcolm Whiting[1], Talia Novos[1], Sabrina Koestier[1], Brett McWhinney[1], Kirsten Hoad[1], Andrew Ellis[1], Trisha Andersen[1]**

*1. Members RCPAQAP Advisory Committee on Biogenic Amines (ACBA), St Leonards, NSW 2065, Australia*

**Introduction**

Plasma free metanephrines (PMET) are a recommended test for the biochemical diagnosis of phaeochromocytoma and paraganglioma. In 2008, The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) introduced the first external quality assurance program for laboratories that measure these analytes. This program has grown internationally with 56 current enrolments. Although nearly all laboratories now utilise mass spectrometry, there are many analytical variables to consider when performing this diagnostic test. ACBA decided to survey laboratories world-wide to determine the degree of harmony that exists for analytical factors associated with PMET performance, and for compliance with clinical practice guidelines[1].

**Methods**

In late 2016, information on analytical variables was requested from all RCPAQAP PMET laboratories (51) in the form of a questionnaire. Details included how plasma samples were collected and stored, analytes measured, the type of chromatography and mass spectrometer in use, sources of calibrators and quality controls, and reference ranges used to interpret results. Survey data were collated and categorised to provide summary tables for analysis.

**Results**

28 participants (55%) from 13 countries across Oceania, Asia, Europe and North America responded to the questionnaire. Questions relating to mass spectrometry parameters displayed the greatest harmony between laboratories, with all respondents indicating identical ionisation source type (Electrospray), ionisation mode (positive) and the use of isotopically labelled internal standards. Pre-analytical and patient preparation practices between laboratories showed the greatest disharmony. The majority of labs (86%) were aware of the Endocrine Society Guidelines.

**Summary**

Although clinical practice guidelines were established in 2014, this survey highlights that practices relating to the measurement of PMET by mass spectrometry show greater harmony during the analytical phase compared to that of the pre-analytical phase amongst 2016 RCPAQAP participants.

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## Manipulation of the MYC/MAX complex influences pheochromocytoma cells pro-metastatic behavior

**Nicole Bechmann[1], Nan Qin[2], Susan Richter[1], Graeme Eisenhofer[1]**

*1. Institute of Clinical Chemistry and Laboratory Medicine , Technische Universität Dresden, Dresden, Germany*

*2. Department of Pediatric Oncology, Hematology, and Clinical Immunology, University Hospital Düsseldorf , Düsseldorf, Germany*

The genetic heterogeneity of pheochromocytomas and paragangliomas (PPGLs) conceivably offers the opportunity to develop an individual treatment strategy for each patient. Therefore, a better understanding of the biomolecular features associated with different mutations is necessary to improve patient care especially after development of metastatic disease. The MYC/MAX (MYC-associated factor X) complex has a central involvement within pathways regulating phenotypic features of PPGLs; however, its role in PPGL progression and malignancy is unknown.

The present study used the rat pheochromocytoma cell line, PC12, lacking *Max* (PC12-EV) and its counterpart cell line re-expressing functional *Max* (PC12-MAX) to investigate how the MYC/MAX complex influences pro-metastatic behavior. Additionally, two small molecule inhibitors targeting MYC/MAX complex formation (MYRA-A) or suppressing *c-Myc* transcription (JQ1) were used. Re-expression of *Max* resulted in an increased n-Myc expression while c-Myc expression was unaffected. Both cell lines showed a comparable doubling time (PC12-MAX: 34.5h; PC12-EV: 32.8h). Treatment with 10 µM JQ1 repressed doubling time significantly (PC12-MAX: 42.3h; PC12-EV: 47.4h), whereas MYRA-A only delayed cell growth in the first 72h. PC12-MAX cells showed significantly lower cell motility (scratch assay, $P<0.001$) compared to control cells. Treatment with JQ1 decreased the motility in both cell lines ($P<0.001$). Moreover, re-expression of *Max* had no impact on migration but reduced the invasion capacity ($P<0.001$), as determined by Boyden Chamber assays. JQ1 diminished cell migration significantly, whereas the invasion capacity of both cell lines was unaffected. In contrast, treatment with MYRA-A had no influence on pro-metastatic behavior.

In conclusion, re-expressing *Max* resulted in a less aggressive cellular phenotype. Moreover, suppression of *c-Myc* transcription by JQ1 diminished pro-metastatic behavior in presence or absence of *Max*. Improved understanding of the MYC/MAX convergence point and involved molecular pathways could be useful for the development of novel treatments against metastatic PPGLs.

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## A giant malignant paraganglioma mimicking hepatic cancer – a case report

**Shi Chen[1], Zheng Pei Zeng[1], Lin Lu[1], Qi Sun[1], Xiao Ping Xing[1]**

*1. Peking Union Medical College Hospital, 1, Shuaifuyuan Wangfujing, BEIJING, China*

**Objective:** To present a rare case of giant malignant paraganglioma in liver mimicking hepatic cancer.

**Methods:** A 40-year-old male with a large hepatic mass was admitted to PUMC hospital in 2017.

**Results:** The patient had a surgical history of left adrenal incidentaloma (7.9cm×6.6cm) diagnosed pheochromocytoma by pathology in 2006. He was admitted to the hospital due to lose weight in 2016. The CT scan showed a large tumor mimicking hepatic cancer in liver（Figure 1）. His Bp was 130/80 mmHg. When he was accepted cholecystectomy and his blood pressure was suddenly elevated into 260/? mmHg during the operation. After then he move forward to PUMC hospital for the diagnosis and treatment in November, 2016. He has paroxysmal hypertension by 220-230/150-160mmHg. The urinary catecholamines and NMNs levels were very high such as DA 503.4µg/24h, NE 1023.9µg/24h, E 5.7µg/24h, NMN 1600.0µg /24h and MN 173.0µg/24h. The PET/CT showed a large aggressive mass (7.8cm×8.0cm×8.4cm) in his liver and $^{131}$I- MIBG image was positive. He was diagnosed as hepatic paraganglioma and taken the surgery by successful resection of tumor and middle liver part (Figure 2) after phenoxybenzamine therapy for 3 weeks. The hepatic malignant paraganglioma was confirmed by pathology and immunohistochemical staining: Melan-A(-), AFP(-), Calretinin(-), CAM5.2(-), CgA(+), CK7(-), Ki-67(index2 %) but SDHB gene mutation was not been found in this patient. At 6 months after operation, his blood pressure is 120/80 and the urinary NE 17.6µg/ 24h, E 2.0µg/24h, DA 159.2µg/24h.

**Conclusions:** The giant malignant paraganglioma located in liver is a very rare neuroendocrine tumor. The correct diagnosis and successfully surgical resection of tumor are important for those patients with paraganglioma. They must be followed up for a long time.

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## Genotype and Clinical Features in Pheochromocytoma/Paraganglioma

**Valeria C de Miguel[1], Andrea L Paissan[1], Patricio García Marchiñena[1], Paula Cuffaro[1], Federico Cayol[1], Gabriela Sansó[2], María L Viale[1], María P Serra[1], Lucas Costa[1], Erica Springer[1], Ester G Scheinfeld[1], Irene Arma[1], Patricia Fainstein Day[1]**

*1. Hospital Italiano de Buenos Aires, San Isidro, BUENOS AIRES, Argentina*

*2. Centro de Investigaciones Endocrinológicas (CEDIE, Hospital de Niños Ricardo Gutierrez, Buenos Aires, Argentina*

**Background:**

Pheochromocytomas (PCC) and paragangliomas (PGL) are neuroendocrine tumors. Nearly 40% of patients with PCC/PGL have germline mutations. Genetic screening algorithims are based on specific clinical features. Phenotype differences from PCC/PGL manifest as different hormonal profile, anatomical localization and risks of recurrence or metastatic disease. According to molecular biology and genetic-based classification PCC/PGL can be divided in three Clusters: Cluster 1a (Krebs Cycle), Cluster 1b (Pseudohypoxic) and Cluster 2 (Kinases signaling).

In order to characterize these groups, we retrospectively reviewed the electronic charts of PCC/PGL patients seen at our hospital (2007 until 2017). We identified 54 PCC/PPGL diagnosed patients. Thirteen (13) had positive genetic testing, one had clinical diagnosis of VHL and two of NF 1 (29.6%).

Sixteen patients were classified in three clusters: Cluster 1a (3 SDHB and 1 SDHD), Cluster 1b (5 VHL), Cluster 2 (2 MEN 2 A, 2 MEN 2 B, 2 NF1 and 1.

| | **Cluster 1a**<br>**N: 4** | **Cluster 1b**<br>**N: 5** | **Cluster 2**<br>**N: 7** |
|---|---|---|---|
| **Mean age at diagnosis** | 44 years old | 24 years old | 32 years old |
| **Male/Female** | 3/1 | 1/4 | 5/2 |
| **Clinical Presentation** | Signs and Symptoms | Signs and Symptoms | Signs and Symptoms (2), Genetic Predisposition (4), Incidentaloma (1) |
| **Biochemical Phenotype** | Noradrenergic phenotype (3), biochemical silent (1) | Noradrenergic phenotype (4) | Adrenergic phenotype (4), biochemical silent (3) |
| **Localization** | Bilateral PCC (1), head and neck PGL (1), abdominal PGL (4) | Bilateral PCC (4), unilateral PCC (1), abdominal PGL (1) | Bilateral PCC (3), unilateral PCC (4) |
| **Size tumor range** | 4-6 cm | 4-6 cm | 2-4 cm |
| **Recurrence/Metastasis** | 2 metastasis | | No |

1 recurrence

**Conclusions:**
Genetic syndromes associated with PCC / PGL are present in a significant proportion of patients. It is essential to carry out the genetic workup and identify particular clinical and biochemical characteristics, risks of recurrence and metastatic disease.

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## SDHAF3 interacts with SDHB: Could an SDHAF3 variant play a role in pheochromocytoma and paraganglioma?

**Trish Dwight[1], Un Na[2], Edward Kim[1], Ying Zhu[3], Anne Louise Richardson[1], Bruce G Robinson[1], Katherine M Tucker[4], Anthony J Gill[5], Diana (Dindy) E Benn[1], Roderick J Clifton-Bligh[1], Dennis R Winge[6]**

*1. Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital and University of Sydney, Sydney*

*2. Departments of Medicine and Biochemistry, University of Utah Health Sciences Center, Salt Lake City, Utah, United States of America*

*3. Hunter New England Health and Royal North Shore Hospital, Sydney, NSW, Australia*

*4. Hereditary Cancer Service, Prince of Wales Hospital, Sydney , NSW, Australia*

*5. Department of Anatomical Pathology and Northern Cancer Translational Research Unit, Royal North Shore Hospital and University of Sydney, Sydney*

*6. Department of Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah , United States of America*

***Background:*** Succinate dehydrogenase assembly factors (SDHAF1-4) have been identified as playing a crucial role in the maturation of individual SDH subunits and assembly of the functioning SDH complex as a whole. ***Methods and Results:*** Through the use of massively parallel sequencing, we identified a variant in SDH assembly factor 3 (*SDHAF3*), c.157T>C (p.Phe53Leu), which was associated with increased prevalence in familial and sporadic pheochromocytoma and/or paraganglioma (6.6%) when compared to normal populations (1.2% [1000 Genomes], p=0.003; 2.1% [Exome Aggregation Consortium], p=0.0063). As this variant was deemed "probably damaging" to protein function (using *in silico* prediction tools [PolyPhen2, SIFT]), we explored the functional consequences of the resulting amino acid change (p.Phe53Leu) in both yeast and human cells. In yeast, introduction of the SDHAF3 variant (p.Phe53Leu) into *Sdh7* null yeast (ortholog of *SDHAF3* in humans) resulted in impaired function, as observed by its failure to restore SDH activity when expressed in *Sdh7* null yeast relative to WT SDHAF3. As SDHAF3 is involved in maturation of SDHB, we tested the functional impact of *SDHAF3* c.157T>C and various clinically relevant *SDHB* mutations on this interaction. Our *in vitro* studies in human cells show that SDHAF3 interacts with SDHB (residues 46 and 242), with impaired interaction observed in the presence of the *SDHAF3* c.157T>C variant. ***Conclusions:*** Our studies reveal novel insights into the biogenesis of SDH, uncovering a vital interaction between SDHAF3 and SDHB. We have shown that SDHAF3 interacts directly with SDHB (residue 242 being key to this interaction), and that a variant in *SDHAF3* (c.157T>C [p.Phe53Leu]) may be more prevalent in individuals with pheochromocytoma and/or paraganglioma, and is hypomorphic via impaired interaction with SDHB.

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## Novel germline *MAX* mutations impair nuclear localisation

**Edward Kim[1, 2], Diana (Dindy) E Benn[1, 2], Anne Louise Richardson[1, 2], Catherine Luxford[1], Margaret Zacharin[3], Emma Duncan[4], Trish Dwight[1, 2], Roderick J Clifton-Bligh[1, 2]**

*1. Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney, NSW, Australia*

*2. University of Sydney, Sydney, NSW, Australia*

*3. Department of Endocrinology and Diabetes, Royal Children's Hospital, Melbourne, VIC, Australia*

*4. Department of Endocrinology, Royal Brisbane Hospital, Brisbane, QLD, Australia*

**Background:** MYC-associated factor X (MAX) is a key protein involved in maintaining balance of cell differentiation, proliferation and apoptosis. Primarily expressed in the nucleus, functional MAX forms a heterodimer with MYC or MXD to regulate transcriptional activity, disruption of which has been reported in various neoplasias. Germline mutations in the gene encoding MAX have been associated with the development of hereditary phaeochromocytoma and paraganglioma (PC/PGL). We recently identified two novel germline *MAX* (NM_002382) mutations, p.Leu64Pro and p.Ala67Asp.

**Objective:** The aim of this study was to assess the biological impact of novel *MAX* mutations, specifically their effect on cellular localisation.

**Method:** Human embryonic kidney cells (HEK293) and rat derived PC cells (PC12) were transfected with DDK-tagged wild type MAX (pCMV6-AC-MAXwt-DDK) or mutant MAX constructs (generated by site-directed mutagenesis). The pathogenic MAX mutant p.Met74Val was used as a positive control. Western blot analyses were used to compare DDK expression between mutant MAX and wild type MAX in the cytosolic and nuclear fractions (enriched using a cellular fractioning kit). Expression of DDK-MAX was normalised to the cytosolic marker (alpha-Tubulin) or nuclear marker (Lamin B1).

**Results:** Protein expression of DDK-tagged wildtype MAX was shown to be predominantly found in the nucleus, as expected. Expression of DDK-MAX was significantly elevated within the cytoplasm in the MAX mutants, p.Leu64Pro and p.Met74Val, when compared to wild type ($n=3$, $p<0.05$). Conversely, in the nuclear compartment expression of the MAX mutants, p.Leu64Pro, p.Ala67Asp and p.Met74Val, were significantly reduced compared to wild type ($n=3$, $p<0.05$).

**Conclusion:** Novel *MAX* germline mutations, p.Leu64Pro and p.Ala67Asp, discovered in Australian PC/PGL cases show impaired nuclear localisation when compared to wild type MAX. Further exploration of the mechanism(s) associated with MAX mutants in PC/PGL may uncover potential strategies for targeted therapy.

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## *SDHA* p.Arg31* is pathogenic but subpenetrant

**Catherine Luxford[2, 1], Anne Louise Richardson[1], Edward Kim[2, 1], Talia Novos[3], Trish Dwight[2, 1], Diana (Dindy) Benn[2, 1], Katherine Tucker[4], Bruce Robinson[2, 1, 5], Chris White[3], Anthony Gill[2, 6], Michael Field[7], Roderick Clifton-Bligh[2, 1, 5]**

*1. Cancer Genetics Laboratory, Kolling Institute, Royal North Shore Hospital, St Leonards, NSW, Australia*

*2. University of Sydney, Camperdown, NSW, Australia*

*3. SEALS North, Department of Clinical Chemistry, Prince of Wales Hospital, Randwick, NSW, Australia*

*4. Hereditary Cancer Centre, Prince of Wales Hospital, Randwick, NSW, Australia*

*5. Department of Endocrinology, Royal North Shore Hospital, St Leonards, NSW, Australia*

*6. Cancer Diagnosis and Pathology Group, Kolling Institute, Royal North Shore Hospital, St Leonards, NSW, Australia*

*7. Northern Sydney Local Health District Familial Cancer Service, Department of Cancer Services, Royal North Shore Hospital, St Leonards, NSW, Australia*

1. Germline and somatic pathogenic *SDHA* variants have been associated with phaeochromocytomas and paragangliomas (PC/PGL)[1].

   The variant *SDHA* c.91C>T predicts premature truncation at codon 31 (p.Arg31*) and is predicted by *in silico* tools to cause loss of normal protein function (either through protein truncation or nonsense-mediated mRNA decay).

   This nonsense variant has been reported in rare individuals with PC/PGL, gastrointestinal stromal tumors (GIST), Carney triad and some non-PGL tumours[1,2,3,4,5,6]. It is also present in the Exome Aggregation Consortium (ExAC) at a frequency of 0.00017 (1/5882), which would predict that it has low penetrance.

   We have identified 2 patients (with paragangliomas) carrying the heterozygous germline *SDHA* c.91C>T, (p.Arg31*) variant. The tumours from these cases demonstrated loss of immunohistochemical staining for both SDHB and SDHA, and metabolomic analysis also confirmed markedly elevated succinate:fumarate ratios (patient 1, 997 ± 320 [n=6]; patient 2, 155 [n=1]), consistent with SDH deficiency.

   Our data supports the hypothesis of the variant *SDHA* c.91C>T being pathogenic but of very low penetrance, possibly due to low rates of somatic *SDHA* inactivation (i.e. LOH). We propose that probands identified with this variant be followed closely for recurrent disease, but that cascade testing of family members should acknowledge the low likelihood of PC/PGL development in asymptomatic carriers.

1. 1.Dénes et al., 2015 2.Korpershoek et al., 2011 3.Rattenberry et al., 2013 4.Pantaleo et al., 2011 5.Niemeijer et al., 2015 6.Boikos et al., 2016

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## *Combined T-SNE and ARACHNE analyses identify root molecular networks underlying subgroups of Pheochromocytoma and Paraganglioma.*

**Kristoffer von Stedingk[1], Ingrid Øra[1]**

*1. Lund University, Lund, Sweden*

Pheochromocytoma (PCC) and Paraganglioma (PGL) are tumours arising from chromaffin cells of the neuroendocrine lineage. They can occur within the adrenal medulla in the case of PCC, where as PGL occurs along the sympathetic or parasympathetic ganglia. Although most often presenting as a benign disease, a subset of patients develop aggressive malignant disease. In such cases, long-term survival is dismal and treatment options are limited. *SDHB*-mutations have displayed association with aggressive disease, however early markers of malignant disease are still lacking.

In this study we performed ARACHNE analysis in combination with gene-based T-SNE dimension reduction on RNA profiling datasets of PCC/PGL in order to identify key transcriptional networks underlying different molecular subtypes of the disease. Interestingly, we identify 3 key dimensions of the disease, consisting of gene-networks regulating epithelial to mesenchymal transition (EMT), xenobiotic metabolism/drug metabolism and finally inflammatory response. These root networks were validated and remained stable across 5 independent PCC/PGL datasets consisting of a total of 538 tumours. Clustering of patients based on these 3 root networks displayed 3 main groups of tumours; (1) those with elevated xenobiotic metabolism, EMT and inflammatory characteristics, (2) those with elevated EMT characteristics alone, and (3) those with low expression of all three of the identified root networks. Group (1) of these classified tumours, displayed a significant association with malignant disease, independent of underlying genetic-mutations including *SDHB*.

Using stable molecular networks to identify new subgroups of PCC/PGLs with varying prognoses may aid not only diagnosing early malignant disease, but also identifying key transcriptional networks that could serve as potential therapeutic targets. Preliminary network analyses reveal *YAP1* as the main central node in regulating the mesenchymal root network in PCC/PGL. Further investigation into the role of YAP1 in functional models could be of biological and clinical interest.

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## SDHC Immunohistochemistry in Pheochromocytoma and Paraganglioma: A Supplementary Tool to Detect Germline *SDHx* mutation

**Zheng Pei Zeng[1], Chuan Shi[2], Da Chun Zhao[3], Han Zhong Li[4], Qi Miao[5], Wen Ling Zhu[6], Shi Chen[2], Lin Lu[2], Zhi Yong Liang[3]**

*1. Peking Union Medical College Hospital, 1, Shuaifuyuan Wangfujing, BEIJING, China*

*2. Endocrinology, Peking Union Medical College Hospital, Beijing*

*3. Pathology, Peking Union Medical College Hospital, Beijing*

*4. Urology, Peking Union Medical College Hospital, Beijing*

*5. Cardiac Surgery, Peking Union Medical College Hospital, Beijing*

*6. Cardiology, Peking Union Medical College Hospital, Beijing*

**Objective** Mutations in succinate dehydrogenase (SDH) related genes (*SDHx*) are most frequently identified genetic variations in pheochromocytomas and paragangliomas (PPGLs), and give rise to hereditary paraganglioma syndromes 1-5. Although it has been encouraged that all PPGL patients should receive genetic testing, universal screening is seldom performed. Recently, SDHB and SDHA immunochemistry study have been proposed as alternative methods in the diagnosis of paraganglioma syndromes, especially SDHB/SDHA immunohistochemistry is a reliable tool to identify patients with SDH-x mutation with an additional value in the assessment of genetic variants. The somatic hypermethylation of SDHC was not investigated, and a germline SDHC mutation and harboring somatic loss of heterozygosity of the SDHC locus paradoxically displayed SDHB immunopositivity. In the current study, we investigated the clinical significance of SDHC immunohistochemistry in PPGLs.

**Design** SDHC and SDHB immunohistochemistry staining were performed on 140 tumor specimens (71 pheochromocytomas, 67 paragangliomas, and 2 metastasis tumor) from 126 PPGL patients. The germline mutation status of 67 patients were previously determined, identifying *SDHA (n*=2), *SDHB (n*=18), *SDHC (n*=2), *SDHD (n*=5), *VHL (n*=2), *RET(n*=7), and *NF1 (n*=1) mutants. Sixteen patients with cardiac paragangliomas were included in the study.

**Results** SDHC expression was absent in 23/30 PPGLs with *SDHx* mutation but preserved in 40/49 tumors without *SDHx* mutation. All 3 *VHL*-mutated tumors stained negative for SDHC, but expressed moderate level of SDHB. Catecholamine secretion, extra-adrenal localization, and malignancy were more common in PPGLs without SDHB expression. Negative SDHB and SDHC immunostaining was observed in 15/16, and 10/16 cardiac paragangliomas, respectively.

**Conclusion** SDHC immunohistochemistry reliably predicts the presence of germline *SDHx* mutation in PPGLs, providing supplementary value to the existing SDHB/SDHA-based immunohistochemical algorithm. Negative SDHB expression in PPGLs indicates malignancy and calls for intense follow-up. SDH-deficiency is a common feature for cardiac paragangliomas, rationalizing *SDH* genetic testing in all cardiac paraganglioma patients.

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## A rare case of cardiac paraganglioma with reoperation and long-time follow-up

**Zheng Pei Zeng[1], Xiao Ping Xing[2], Shi Chen[2], Lin Lu[2], Qi Sun[2], Qi Miao[3]**

*1. Peking Union Medical College Hospital, 1, Shuaifuyuan Wangfujing, BEIJING, China*

*2. Endocrinology, Peking Union Medical College Hospital, Beijing*

*3. Cardiac Surgery, Peking Union Medical College Hospital, Beijing*

**Objective:** Paragangliomas are rare neuroendocrine tumors arising from the ganglia of the sympathetic or parasympathetic nervous system. Here we report a rare case with cardiac paraganglioma successfully treated by reoperations.

**Methods:** A 53-year-old male with cardiac paraganglioma was admitted to PUMCH in 2017.

**Results:** He was admitted to the cardiovascular hospital with paroxysmal headache, palpitation, profuse sweating and hypertension in 2003, and the CT scan showed intra-atrial tumor. He had had an operation on his cardiac tumor which grow into left atrium(6*7cm) and right atrium(3*4cm) during cardiopulmonary bypass in 2003 without α-block peraperation. The pathology showed paraganglioma with immunohistochemical staining: CgA（+）, Syn（+）, Actin(-),Des(-),CD34(-). The blood pressure return nomal after the operation but PET/CT still showed positive mass in left atrium region. Then, He was admitted to PUMCH in December, 2003. The lab examination showed 24h urinary DA 239.68-326.76ug/24h,NE 56.67-100.56ug/24h,E 2.43-5.50ug/24h. CT scan showed a low density mass(3.4*3cm) in left atrium, near inferior vena cava with heterogeneous enhancement after intravenous contrast agent. Octreotide scan revealed positive with MIBG scan negative . From 2003 to 2015, he was followed up in PUMCH. The lab examination and CT scan of the patient were shown in Table1 and Fig1. In 2015, he had had another operation after the prepration of phenoxybenzamine for one month. The lab examination revealed 24h Urinary DA 191.87-228.38 ug/24h,NE 23.71-39.15ug/24h,E 2.76-3.26ug/24h after the operation. His blood pressure and cardiac CT scan remain normal until now.

**Conclusions:** As a rare neuroendocrine tumor, cardiac paraganglioma often require reoperation to cure, and it is important for a long-time follow-up.

Table1: The lab examination of the patient in his follow-up

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## Pheochromocytoma associated with systemic lupus erythematosus in a patient with multiple endocrine neoplasia 2A: a case report.

**Zheng Pei Zeng[1], Lin Lu[2], Shi Chen[2], Chuan Shi[2], An Li Tong[2], Wei Gang Yan[3], Han Zhong Li[3]**

*1. Peking Union Medical College Hospital, 1, Shuaifuyuan Wangfujing, BEIJING, China*

*2. Endocrinology, Peking Union Medical College Hospital, Beijing*

*3. Urology, Peking Union Medical College Hospital, Beijing*

**Objective:** Pheochromocytoma is a rare disease. The systemic lupus erythematosus (SLE) could be a manifestation of paraneoplastic syndrome of pheochromocytoma. we report a MEN 2A case with right adrenal pheochromocytoma and medullary thyroid cancer associated with a newly diagnosed SLE.

**Methods:** The clinical manifestation, lab results and imaging were described and follow-up was carried out to the changes of autoantibody after surgery.

**Results:** A 52-year-old female was admitted to department of endocrinology in PUMCH with paroxysmal headache, palpitation for 11 years in 2016. She didn't monitor blood pressure. The CT scan showed a 4.6×4.7cm left adrenal mass in the local hospital. Then she was sent to operation without further examination and medical preparation in local hospital. The blood pressure raised to the 240/180mmHg when the surgeon detected the tumor. The operation was discontinued and the patient was transferred to PUMC Hospital. 24h urine catecholamine revealed normal (NE 21.35ug, E 31.06ug↑, DA 133.94ug). The MIBG and $^{99m}$Tc-octreotide scanning showed high uptake in the left adrenal region. The thyroid ultrasonography revealed 3.0×1.8×1.8cm hypoechoic solid nodule in the left thyroid. The nodule had irregular shape, unclear boundary and multiple microcalcifications. The several enlarged cervical lymphnodes were detected with the disappearance of normal medulla structure. The ANA 1:640, decreased C3. C4 and several positive autoantibodies were found due to low count of white blood cell. The SLE was diagnosed by the rheumatologist. After the management of phenoxybenzamine, the operation of pheochromocytoma and thyroid were carried out successively. The pathology revealed the MTC of the thyroid. MEN2A could be diagnosed.

**Conclusions:** SLE accompanying with the pheochromocytoma was reported very rarely. After the removal of pheochromocytoma, the improvement of SLE could be attained in some cases. The SLE was considered it could be one of the rare paraneoplastic syndrome of the pheochromocytoma.

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## A giant malignant paraganglioma mimicking hepatic cancer – a case report

**Zheng Pei Zeng[1], Shi Chen[2], Lin Lu[2], Qi Sun[2], Xiao Ping Xing[2]**

*1. Peking Union Medical College Hospital, 1, Shuaifuyuan Wangfujing, BEIJING, China*

*2. Endocrinology, Peking Union Medical College Hospital, Beijing*

**Objective:** To present a rare case of giant malignant paraganglioma in liver mimicking hepatic cancer.

**Methods:** A 40-year-old male with a large hepatic mass was admitted to PUMC hospital in 2017.

**Results:** The patient had a surgical history of left adrenal incidentaloma (7.9cm×6.6cm) diagnosed pheochromocytoma by pathology in 2006. He was admitted to the hospital due to lose weight in 2016. The CT scan showed a large tumor mimicking hepatic cancer in liver（Figure 1）. His Bp was 130/80 mmHg. When he was accepted cholecystectomy and his blood pressure was suddenly elevated into 260/? mmHg during the operation. After then he move forward to PUMC hospital for the diagnosis and treatment in November, 2016. He has paroxysmal hypertension by 220-230/150-160mmHg. The urinary catecholamines and NMNs levels were very high such as DA 503.4μg/24h, NE 1023.9μg/24h, E 5.7μg/24h, NMN 1600.0μg /24h and MN 173.0μg/24h. The PET/CT showed a large aggressive mass (7.8cm×8.0cm×8.4cm) in his liver and $^{131}$I- MIBG image was positive. He was diagnosed as hepatic paraganglioma and taken the surgery by successful resection of tumor and middle liver part (Figure 2) after phenoxybenzamine therapy for 3 weeks. The hepatic malignant paraganglioma was confirmed by pathology and immunohistochemical staining: Melan-A(-), AFP(-), Calretinin(-), CAM5.2(-), CgA(+), CK7(-), Ki-67(index2 %) but SDHB gene mutation was not been found in this patient. At 6 months after operation, his blood pressure is 120/80 and the urinary NE 17.6μg/ 24h, E 2.0μg/24h, DA 159.2μg/24h.

**Conclusions:** The giant malignant paraganglioma located in liver is a very rare neuroendocrine tumor. The correct diagnosis and successfully surgical resection of tumor are important for those patients with paraganglioma. They must be followed up for a long time.

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## Functional-structural mapping of *TMEM127* Mutation

**Patricia Dahia**[1]

*1. University of Texas Health Science Center, Texas, United States*

S. K. Flores, Y. Deng, Z-M. Cheng, Y. Qin, P. L.M. Dahia. Germline mutations in the transmembrane protein-encoding gene, *TMEM127*, have been associated with pheochromocytomas (PHEOs), highly hereditary tumors of the adrenal medulla. The aim of the study was to characterize the functional consequences of several germline *TMEM127* mutations detected in patients with PHEOs by investigating the subcellular localization and protein stability of the resulting mutant TMEM127 proteins. GFP-tagged wild-type (WT) and mutant TMEM127 plasmids were generated and transiently transfected into HEK293FT cells. Cells were collected for confocal images and/or Western blot at 24, 48 and 72 hours. TMEM127-TMEM127 interactions were investigated by use of multiple tags and pulldown assays. Missense mutations (N=4) that occurred outside of putative transmembrane domains resulted in mutant proteins that retained a punctate pattern similar to the WT TMEM127 indicative of retention of membrane localization. Missense mutations (N=5) and frameshift mutations (N=2) that occurred within or disrupted putative transmembrane domains resulted in mutant proteins with a diffuse, cytoplasmic pattern suggestive of loss of membrane localization. Moreover, mutant proteins with a diffuse, cytoplasmic pattern were unstable and rapidly degraded within 72 hours compared to WT TMEM127. We identified TMEM127-TMEM127 interactions suggesting that this protein forms dimers or multimers. Membrane localization of TMEM127 is necessary for its stability and, likely, its function. Loss of membrane localization is associated with mutations disrupting putative transmembrane domains. Although mutations occurring outside of putative transmembrane domains retain membrane localization, another function, such as binding to an associated protein, may be affected and requires further investigation. Furthermore, our results support a novel, 4th functional transmembrane domain and TMEM127-TMEM127 interactions, suggesting that dimerization (or multimerization) may be relevant for its function.

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## Variable presentation in patients with *tmem127* mutations

**Diana (Dindy) Benn**[1, 2], **T Dwight** [1, 2], **E Kim**[1, 2], **W Inder**[3], **A Crook**[4], **A Luxford**[1], **AL Richardson**[1], **BG Robinson**[1, 2], **M Field**[3], **RJ Clifton-Bligh**[2]

*1. Kolling Institute of Medical Research, St Leonards, NSW, Australia*

*2. University of Sydney , NSW, Australia*

*3. Department of Diabetes and Endocrinology, Princess Alexandra Hospital , NSW, Australia*

*4. Department of Cancer Services, Royal North Shore Hospital, Sydney, NSW, Australia*

**Introduction:** It is recommended that genetic testing be offered to all patients with familial and apparently sporadic pheochromocytoma (PC) and/ or paraganglioma (PGL) as germline mutations may be detected in up to 30 % of cases. In addition to *VHL, RET, NF1, SDHA, SDHB, SDHC, SDHD SDHAF2, MAX* and *FH* testing, *TMEM127* mutation analysis has been added to decision algorithms which assist in prioritizing genetic testing, a lengthy and costly process in PC/PGL patients. Typically most patients with *TMEM127* mutations present with unilateral disease (bilateral in 28%), almost exclusively benign adrenal PC with an average age of 41.5 years and few cases with metastases. We report here four novel *TMEM127* variants of likely pathogenic significance. **Patients.** Patient 1(P1) presented at age 39 years (having experienced relevant symptoms for many years) with no family history, raised catecholamines and underwent surgery for bilateral PCs; P2 had an adrenal PC removed at age 52 years; P3 with a family history of renal cell carcinoma, underwent surgery for adrenaline- and noradrenaline- secreting bilateral PC at age 54 years; and P4, a 57 year old was diagnosed with a composite PC and ganglioneuroma with a history of hyperparathyroidism. **Methods.** Genomic DNA from the four patients was sequenced by a combination of Sanger and/or targeted massively parallel sequencing. All potentially pathogenic variants were verified by Sanger sequencing (of two samples) and clinical significance of variants predicted by *'in silico'* programs. None of the four patients harboured *RET, VHL, SDHD or SDHB* mutations. **Results.** Four novel *TMEM127* variants were identified each with a different clinical presentation. The following variants were identified: exon 4 insertion; deletion of 3 nucleotides in exon 4; deletion of 4 nucleotides in exon 2; missense mutation in exon 2. **Conclusion.** This report expands the genetic and phenotypic spectrum of *TMEM127* mutation-associated PC/PGL.

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## Biochemical diagnosis of pheochromocytoma: Plasma free versus urinary deconjugated and urinary free normetanephrine, metanephrine and methoxytyramine

**Graeme Eisenhofer**[1]

*1. Techniche Universität Dresden, Dresden, Germany*

**Background:** Measurements of plasma free or urinary fractionated metanephrines are both recommended for diagnosis of pheochromocytoma and paraganglioma (PPGL). What test offers optimal diagnostic accuracy, whether urinary free metabolites offer advantages over deconjugated metabolites and the relative utilities of measurements of methoxytyramine remain unclear.

**Methods:** LC-MS/MS measurements of urinary deconjugated and plasma and urinary free metanephrines and methoxytyramine were prospectively carried out in 2093 patients with suspected PPGLs, which were confirmed in 210 patients.

**Results:** Areas under receiver-operating characteristic (ROC) curves for diagnosis of PPGLs using normetanephrine and metanephrine did not differ between plasma and urinary free metabolites (0.984 vs 0.975), but were higher (P=0.0051) for plasma than urinary deconjugated metabolites (0.970). Areas under ROC curves were higher (P<0.0001) for plasma free methoxytyramine (0.883) than urinary free (0.713) and deconjugated (0.705) methoxytyramine. Thus, with addition of methoxytyramine, areas under ROC curves were higher (P<0.02) for plasma free (0.991) than both urinary free (0.975) and deconjugated (0.970) metabolites. Using optimized cut-offs, measurements of plasma free metabolites offered higher diagnostic sensitivity (98.6%vs94.9%vs94.7%; P<0.04) and specificity (95.2%vs92.4%vs88.5%; P<0.0001) than urinary free and deconjugated metabolites. Sensitivity between urine tests did not differ but specificity was higher (P<0.001) for tests of urinary free than deconjugated metabolites.

**Conclusions:** Diagnosis of PPGLs using urinary free metanephrines rather than routinely used deconjugated metabolites provides advantages of fewer false-positive results. With inclusion of methoxytyramine, which shows limited diagnostic utility for measurements in urine, the plasma test shows superior diagnostic performance than tests of both urinary free and deconjugated metabolites

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## A case of bilateral ACTH-secreting pheochromocytomas

**Anli Tong**[1], **Fen Wang**[1], **Chunyan Li**[1], **Jian Sun**[2], **Ailing Song**[3], **Yuxiu Li**[1]

*1. Department of Endocrinology, Ministry of Health , China*

*2. Department of Pathology, China*

*3. Clinical Laboratory, Chinese Academy of Medical Sciences, Beijing, China*

**Introduction**: Ectopic ACTH syndrome is usually caused by pulmonary and bronchial tumors and rarely by pheochromocytoma. To date, most ACTH secreting pheochromocytomas reported are unilateral except two. Presented here is a third bilateral ACTH-secreting pheochromocytoma.

**Patients and Methods:** A 54-year-old male presented with hypertension and bilateral adrenal tumors. He denied having classic cushingoid features, or paroxysmal attacks of headache, palpitation and sweating. He was diagnosed with bilateral ectopic ACTH-secreting pheochromocytomas on the basis of biochemical and imaging findings. Then the tumors were removed, and pathologically diagnosed as pheochromocytomas. whole-exome of genomic DNA was *sequenced,* and secretion of ACTH and catecholamine were observed both in vivo and in vitro.

**Results:** Whole-exome *sequencing showed that the* 19 pheochromocytoma-related genes were all normal. The pheochromocytomas on both sides were all negatively stained for ACTH. But the ACTH concentration in the tumor tissue homogenates was much higher than that in other three pheochromocytomas without ACTH secretion. Electron microscopy identified two kinds of neuroendocrine cells in the tumor tissue. In primary culture of the pheochromocytoma cells, ACTH secretion was inhibited by mTOR inhibitor, AZD8055.

**Conclusion:** We reported a third bilateral ectopic ACTH-secreting pheochromocytoma. mTOR inhibitor could inhibited ACTH secretion in vitro.