A study of succinate dehydrogenase deficient tumourigenesis: From functional assessment of variant pathogenicity to the identification of new disease biomarkers

Submitted for the degree of Doctor of Philosophy by
Ruth Therese Casey (ID: 303313010)
Wolfson College

Research conducted at the Department of Medical Genetics,
University of Cambridge
Declaration:

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text.
Abstract:

A loss of function of the citric acid cycle enzyme complex succinate dehydrogenase (SDH) is associated with a predisposition to a spectrum of tumourigenesis including phaeochromocytoma, paraganglioma (1) (PPGL), gastrointestinal stromal tumours (GIST) (2), renal cell carcinoma (RCC) (3) and pituitary adenomas (4). Pathogenic variants in each of the four genes (SDHx) encoding the four sub-components of this complex (SDHA/B/C/D) have been associated with tumourigenesis. Germline pathogenic variants in SDHB account for up to 50% of patients with malignant PPGL and a 5 year survival of less than 50% in those with malignancy (5). Most SDHx variant carriers require life long surveillance for tumour development (6) but predicting malignant disease is challenging and histology is of limited assistance in this prediction. The advent of next generation sequencing (NGS) has been influential in this field of inherited neoplasia allowing more rapid and accurate identification of pathogenic variants in the SDHx genes. However the increased throughput achieved with NGS methodology has yielded more variants of uncertain significance in these genes which require additional assessment. New diagnostic adjuncts such as SDHB immunohistochemistry(7), have provided additional prognostic information and prediction of malignant risk but further biomarkers are needed. Furthermore there is a lack of effective treatments for malignant disease associated with SDHx variants (8) (9). This ‘multi-omics’ investigation has provided new insights into genotype-phenotype correlations in SDH deficient disease and has facilitated the translation of new techniques into clinical utility which will aid SDHx variant interpretation. This study has evaluated novel disease biomarkers and potential therapeutic targets in SDH deficient tumours and has facilitated further progress in the pursuit of a precision medicine model of care for patients with rare inherited neoplasia syndromes.
Contents

Title page..................................................................................................................................................1

Abstract......................................................................................................................................................3

Abbreviations ...............................................................................................................................................7

Chapter 1: Introduction .................................................................................................................................9

Chapter 2: General methods.......................................................................................................................49

Chapter 3: In silico protein prediction models and SDHB Immunohistochemistry….. .........................................................................................................................................................62

Chapter 4 : Investigating the clinical and molecular features of Renal and phaeochromocytoma/paraganglioma a tumour syndrome .................................................................106

Chapter 5: Investigating the somatic mutational landscape of hereditary and sporadic phaeochromocytoma and paraganglioma............................................................................................135

Chapter 6: Investigating the clinical applications of ex-vivo metabolomic profiling ........................................................................................................................................................................169

Chapter 7: Translating in vivo metabolome analysis of SDH deficient tumours into clinical utility ....................................................................................................................................................206

Chapter 8: Investigating the role of SDHC epigenetic silencing in the pathogenesis of phaeochromocytoma/paraganglioma and GIST ..........................................................................................237

Chapter 9: Discussion .................................................................................................................................269

Chapter 10: Bibliography ...........................................................................................................................283

Chapter 11: Appendix .................................................................................................................................328
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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
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<td>Alanine</td>
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<tr>
<td>α-ketoglutarate</td>
<td>alpha-ketoglutarate</td>
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<td>Arg</td>
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<td>Asn</td>
<td>asparagine</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>cDNA</td>
<td>complimentary DNA</td>
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<tr>
<td>CpG</td>
<td>5’—C—phosphate—G—3’</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>FFPE</td>
<td>formalin fixedx paraffin embedded</td>
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<td>fresh frozen</td>
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<td>GADPH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GIST</td>
<td>gastrointestinal stromal tumour</td>
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<td>Gly</td>
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<td>glutamine</td>
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<td>fumarate hydratase</td>
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<td>hypoxia inducible factor</td>
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<td>high resolution magic angle spinning</td>
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<td>H1-MRS</td>
<td>Magnetic resonance spectroscopy</td>
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<tr>
<td>Ile</td>
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<td>MAX</td>
<td>MYC associated factor X</td>
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<td>MEN</td>
<td>multiple endocrine neoplasia</td>
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<td>MDH</td>
<td>malate dehydrogenase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase</td>
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<td>MIBG</td>
<td>metaiodobenzylguanidine</td>
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<td>MRI</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>Neurofibromatosis</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PIK3CA</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha</td>
</tr>
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<td>PNMT</td>
<td>phenyl ethanolamine-N-methyltransferase</td>
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<tr>
<td>RAPTAS</td>
<td>Renal and phaeochromcytomat/paraganaglioma a tumour syndrome</td>
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<tr>
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<td>retinoblastoma</td>
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<td>RCC</td>
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<td>von Hippel Lindau</td>
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<td>2-HG</td>
<td>2-hydroxyglutarate</td>
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Chapter 1

Introduction
Introduction

1.0 A brief introduction into the role of medical genetics in Oncology
   a) Genetic testing for inherited cancer syndromes
   b) Cancer genotyping
   c) Epigenetics and gene expression in cancer

1.11 Discovery of phaeochromocytoma/paraganglioma (PPGL) predisposition genes

1.12 The role of the succinate dehydrogenase (SDHx) genes in normal cellular function

1.13 Functional impact of pathogenic SDHx variants on the SDH complex

1.14 Pathways implicated in SDHx related tumorigenesis
   a) Succinate as an ‘oncometabolite’
   b) Hypoxia signaling
   c) Hypermethylation

1.15 Genotype-phenotype correlations
   a): SDH deficient PPGL
   b): SDH deficient gastrointestinal stromal tumours (GIST)
   c): SDH deficient renal tumours
   d): SDH deficient pituitary adenomas
   e): Rare SDH deficient tumours

1.16 Disease penetrance with germline SDHx variants

1.17 The role of next generation sequencing in the detection of germline SDHx variants

1.18 Surveillance protocols for pathogenic SDHx variant carriers

1.19 Treatment options for metastatic SDH deficient disease
   a): Treatment options for SDH deficient PPGL
   b): Treatment options for SDH deficient GIST
   c): Treatment options for other SDH deficient tumours

1.2 Barriers to optimal clinical practice
1.21 Interpretation of variants of uncertain significance

1.22 Limited effective biomarkers to predict and follow malignant SDH deficient tumours

1.23: Study hypotheses

1.24: Aims of PhD study
1.0 A brief introduction into the role of medical genetics in Oncology

Cancer remains one of the leading causes of death globally, with over 8 million cancer related deaths worldwide recorded by the World Health Organisation in 2012. However, cancer survival rates are improving and data from Cancer Research UK (http://www.cancerresearchuk.org) indicate that survival from cancer has doubled over the past 40 years. Improved survival is multifactorial owing to: improved public health awareness, ii) early detection and advanced screening programmes and iii) improved treatment strategies.

Advances in the field of genetics have resulted in a better understanding of inherited disease and cancer genetics. The concept that cancer might result from a genetic predisposition was first observed by the French neurosurgeon Paul Broca in the nineteenth century, when he recognised that breast cancer seemed to run in his wife’s family (10) and almost fifty years later a German scientist, Theodor Boveri, first implicated chromosomal abnormalities in tumourigenesis (10). In the present day, cancer is understood to develop as a result of unregulated cell growth, migration and cell death and that failure of these cell regulatory processes can arise from acquired variation in two categories of genes; i) proto-oncogenes and ii) tumour suppressor genes.

i) Proto-oncogenes

Proto-oncogenes are highly conserved genes and expressed under normal conditions of cell growth or repair such as embryogenesis and wound healing. Proto-oncogenes encode proteins such as growth factors with tyrosine kinase activity and transcription factors, which are crucial for regulated cell growth, migration and programmed cell death (11). Proto-oncogenes can be activated to cancer initiating ‘oncogenes’ by point variants, structural DNA
rearrangements and oncogenes can be also transduced into host DNA by acute transforming retroviruses (11).

Oncogenes were first identified indirectly through the study of retroviruses, which have an RNA genome but rely on the host cell DNA for replication, thus resulting in cell transformation or tumourigeneses in vitro (12). The mechanism by which a retrovirus integrate their genome with the host DNA is termed ‘insertional mutagenesis’ and this mechanism of insertional mutagenesis has been widely exploited for cancer gene discovery (13).

Another mechanisms, which affects the expression or function of proto-oncogenes include chromosomal translocations. This cytogenetic aberration is best exemplified by the reciprocal translocation between chromosome 9 and chromosome 22, which results in the fusion of the c-abl proto-oncogene (chromosome 9) with the bcr locus on chromosome 22 and drives the development, maintenance and progression of chronic myeloid leukemia (14).

ii) Tumour suppressor genes

Following the discovery of oncogenes, the concept of ‘anti-oncogenes’ was postulated, without definitive evidence in the scientific community for a number of years until 1969, when Alfred Knudson published his work on the kinetics of developing sporadic and familial retinoblastoma (15). Knudson proposed a ‘two-hit’ model of carcinogenesis, meaning that for inherited cancers, the first hit is in the germline and that for sporadic cancers, both hits occur in the same somatic cell (15). This ‘two-hit’ model of tumourigenesis was later supported by the finding that 5-10% of hereditary retinoblastoma cases harboured a constitutional deletion of chromosome band 13q14 and this revealed the location and led to the subsequent cloning of the retinoblastoma (RB1) gene (16).
Tumour suppressor genes are now understood to be important regulators of cell growth and programmed cell death and inactivation of these gatekeeper genes are known to promote tumourigenesis (17). Inactivation of tumour suppressor genes is mediated by a number of mechanisms including intragenic mutations, copy number variations including deletions, insertions, rearrangements and epigenetic aberrations.

Identification of tumour suppressor genes can also be achieved through a number of mechanisms. Linkage analysis involves the mapping of tumour suppressor genes and is most effective for the mapping of rare alleles that result in highly penetrant cancer syndromes (18). Another mechanism for localising tumour suppressor genes is loss of heterozygosity studies. Loss of heterozygosity (LOH) refers to the loss of the normal allele at a heterozygous locus in cancer cells, as is required for tumourigenesis according to Kundson’s two hit hypothesis. In an individual with a germline variant in a tumour suppressor gene, LOH may occur due to a deletion, mitotic recombination or non-disjunctive chromosomal loss (19). LOH studies were responsible for the successful localisation of a number of tumour suppressor genes including the Menin gene implicated in multiple endocrine neoplasia type 1 (MEN1) (20).

Advances in cancer genetics has played an important role in improved cancer survival rates and Bert Vogelstein and his partner Kenneth Kinzler are accepted as pioneers in the field of cancer genetics, as their motivation to empty cancer clinics led to the breakthrough in the late 1980’s, that the TP53 gene was a tumour suppressor gene rather than an oncogene and that mutations in this gene affect the protective function of the gene and predisposed to the development of cancer (21). We now accept that inherited pathogenic genetic variants are causative in 5-10% of cancers and over 50 hereditary cancer syndromes have now been described. This era of gene discovery has prompted the development of a comprehensive referral guideline by the American College of Medical Genetics and Genomic Medicine in
2015, in order to raise awareness among physicians regarding the indications for referral for cancer predisposition assessment with the major factors prompting investigation for pathogenic germline variants being; family history and features of specific familial cancer syndrome (22). The aim of diagnosing familial cancer syndromes is threefold; i) preventative strategies and opportunity for early intervention in unaffected relatives, ii) appropriate surveillance for synchronous and metachronous tumours in affected individuals and iii) opportunities for the development of targeted or personalised treatment options in those with cancer based on a better understanding of the molecular drivers.

**Figure 1.1:** Roadmap of common cancer predisposition gene discoveries over the past two centuries. Adapted from (22)
1.10 a): Genetic testing for inherited cancer syndromes

Progress in the field of medical genetics was largely assisted by a simultaneous development of new gene sequencing technologies. Sanger sequencing was first invented in 1977 by Frederick Sanger and colleagues (23) and has been universally adopted by the genetics community to the present day. Sanger sequencing together with polymerase chain reaction (PCR) methods (24), facilitated sequencing of the human genome in 2001(25). Four years later, the first commercially available next generation sequencing (NGS) platform was launched (25) and this has revolutionised modern medical genetics practice. The rapid throughput achieved with NGS enabled the barrier of the $1000 genome to be broken in 2014 (26) and over the past decade further advancements in NGS technologies including sequencing speed, read length, coupled with a significant reduction in cost (26) has resulted in NGS technology being the preferred sequencing method in most clinical diagnostic laboratories. Clinical NGS assays for possible inherited cancer predisposition often utilise multigene panels of cancer predisposition genes making it a timely and cost effective diagnostic tool. The value of NGS multi-gene panel testing in suspected hereditary cancer syndromes was elegantly demonstrated by Pritchard and colleagues in a study of subjects with suspected Lynch syndrome in 2012 (27) and has been widely adopted in many diagnostic cancer genetic services to date. In the future if reductions in the cost of sequencing technologies continues, genome-wide analysis through whole exome sequencing (WES) or whole genome sequencing (WGS) may become more common place in the clinical setting as these technologies will provide a more comprehensive sequencing method including the provision of additional information on non-coding regions of the genome and structural variation in the case of WGS.
1.10 b): Cancer genotyping:

It is now understood that the autonomous unregulated growth of cancer cells is driven in part by an accumulation of acquired genetic variants that are unique to the cancer genome and referred to as somatic variants (28). These somatic variants unique to the cancer cell genome can include several distinct classes of DNA sequence change and regardless of the structural nature, each somatic variant can be further classified according to its proposed effect on cancer development. Somatic variants are referred to as ‘driver’ variants, if they are believed to confer specific growth advantage on the cancer cells. The remainder of somatic variants are ‘passengers’ that do not confer direct clonal growth advantage, but may play an indirect or cumulative role in tumorigenesis. Studying the cancer genome offers an opportunity to gain further insights into the molecular and biological processes implicated in cancer, however a key challenge in the delivery of a precision based model of care is to distinguish driver from passenger variants. One strategy is to prioritise variants in known predisposition oncogenes or to identify key mutational signatures association with variants that are positively selected by cancer cells (29). Advances in databases and cataloguing of identified somatic variants such as the Catalogue of Somatic Mutations in Cancer (COSMIC) (30) have facilitated the approximation that 1.6% of protein-coding genes in the human genome are subject to recurrent somatic driver variants in cancer cells (29). A number of these discoveries serve as a paradigm for precision oncology, such as; i) the discovery of somatic activating variants in IDH1 (31), which has revolutionized the management approach in patients with low grade gliomas harboring this variant (32), ii) the identification that chromosomal translocations in chronic myeloid leukemia lead to the development of a fusion oncogene BCR-ABL and the subsequent development of Imatinib, a targeted inhibitor of the constitutively active BCRABL tyrosine kinase (33). Therefore cancer genotyping has the potential to identify molecular biomarkers, which can have diagnostic, prognostic and
therapeutic utility and this molecular revolution has influenced a paradigm shift in the approach to a number of cancers including lung cancer and breast cancer over the past century (34)(35). Despite major advances in the molecular profiling of more common cancer types, rare cancers still pose a clinical challenge as relatively little is known about their molecular underpinnings.

1.10 c): Epigenetics and gene expression in cancer

The processes implicated in regulation of cellular gene expression remains a challenge for the genetics community and this challenge extends to the field of cancer genetics. Epigenetics refers to a specific form of modification, which affects DNA conformation and gene expression without affecting the DNA sequence (36). Epigenetic modifications which affect gene expression include; i) binding of proteins such as methyl binding proteins or histones to DNA, ii) loss or gain of methyl groups to DNA bases and iii) mechanisms mediated by non-coding RNA’s (37).

One of the most studied epigenetic markers in cancer genetics is DNA methylation. It is understood that DNA hypomethylation is often a hallmark in early carcinogenesis and can lead to chromosomal instability (38). On the other hand DNA hypermethylation particularly in the promoter region of a gene can down regulate gene expression and is associated with tumour development particularly if DNA hypermethylation silences the expression of tumour suppressor genes (39), as it is postulated that DNA hypermethylation affects gene transcription as binding of the methyl group prevents successful binding of key transcription factors to the gene promoter. An example of the value of exploring this epigenetic modification in oncological disease is the discovery that the DNA repair gene MGMT is downregulated in brain tumours as a result of promoter region DNA hypermethylation (40).
This discovery was followed by the realization that tumours that have down regulated $\textit{MGMT}$ are more sensitive to radiotherapy and chemotherapy with temozolomide (40), therefore the discovery of this epigenetic aberration has guided clinical decisions and management for patients with these tumours.

The three DNA methyltransferase enzymes (DNMT 1-3) are responsible for regulating DNA methylation in eukaryotic cells (41) and DNMT1 is believed to play a crucial role in regulating DNA methylation in cancer cells. As DNA methylation is a potentially reversible modification, therapeutic targeting in oncological disease has also focused on the development of agents which block these enzymes such as the de-methylation agent 5-Azacytidine, which was initially used in the management of myelodysplastic syndrome (42) and this has paved the way for the development of next generation DNMT inhibitors (43).

Although localized changes in methylation within the genome have long since been appreciated, it is also increasingly recognised that these epigenetic alterations can be genome wide in cancer (44) and indeed emerging data would suggest that the entire epigenome is fundamentally abnormal in cancer, highlighting the potential for combination therapies aimed at rectifying multiple epigenetic modifications in the future (45).
1.11: Discovery of phaeochromocytoma and paraganglioma (PPGL) predisposition genes

Prior to the turn of this century, it was widely accepted that a phaeochromocytoma was a catecholamine secreting adrenal tumour that clinically followed a rule of ‘tens’; i) 10% were bilateral, ii) 10% were malignant, iii) 10% were extra adrenal and 10% were familial. Familial occurrence of phaeochromocytoma was first recognised by Chase et al in 1933(46) and almost fifty years later the co-occurrence of phaeochromocytoma and its extra adrenal counterpart; paraganglioma (PPGL) in the same kindred was reported (47). Those tumours that were familial were associated with hereditary syndromes such as Neurofibromatosis(48), Multiple Endocrine Neoplasia type 2 (MEN2)(49) and Von Hippel Lindau syndrome (VHL) (50). Each of these syndromes predispose to the development of bilateral phaeochromocytoma but extra adrenal tumours are rarely encountered and typically each of these syndromes are associated with other characteristic phenotypic features.

Neurofibromatosis was first described by Von Recklinghausen in 1882(48) as a hereditary syndrome characterised by neuro-ectodermal abnormality as well as systemic manifestations affecting bone, nervous system, eyes and other sites. In 1990, the \textit{NF1} gene was localised to chromosome 17 and further studies facilitated cloning and additional characterisation of the \textit{NF1} gene(48). In the present day a diagnosis of neurofibromatosis is guided by strict clinical criteria and genetic testing is rarely required (51). The development of a phaeochromocytoma is rare with this syndrome, affecting 0.1%-6% of all patients with neurofibromatosis type 1 (52).

In the early 1970’s, Sizemore and his colleagues described the syndrome of MEN2 and sub-classified the syndrome into MEN2A and MEN2B. MEN2A syndrome is associated with a predisposition to medullary thyroid carcinoma, phaeochromocytoma and
hyperparathyroidism, and MEN2B predisposes to the medullary thyroid carcinoma and phaeochromocytoma, but is also associated with mucosal neuromas and mesodermal abnormalities (53). In 1993, Lois Mulligan et al, working in the laboratory of Bruce Ponder in Cambridge, was the first to demonstrate that the RET oncogene was responsible for the development of MEN2 syndrome (49). In more recent years, increasing clinical data has led to the realization that very specific genotype-phenotype correlations exist in MEN2A syndrome and that variants in codon 634 and 918 of the RET gene are most commonly associated with the development of phaeochromocytoma, but overall inherited pathogenic variants in the RET gene predict a 30-50% lifetime risk of developing a phaeochromocytoma (56)(55).

In 1894 Treacher Collins first described familial retinal angiomatosis in a brother and sister and ten years later in 1904 Eugen Von Hippel again described the occurrence of retinal angiomas in two siblings (56). It was a Swedish pathologist, Arvid Lindaue who first made the association between retinal angiomata and the central nervous system tumours and in 1936 the syndrome of von Hippel Lindau was coined (56). Thirty years later in 1964, Melmen, Rosen and colleagues described the first clinical criteria for VHL (57) and almost thirty years later again the VHL gene was mapped to the short arm of chromosome 3 (50). VHL is recognised as a multiple tumour syndrome including; renal cell carcinoma, hemangioblastomas of the eye and central nervous system, renal and pancreatic cysts, pancreatic neuroendocrine tumours and phaeochromocytoma (58). Genotype-phenotype correlations have also emerged for the VHL gene, including the discovery that patients with large deletion or truncating variants in the VHL gene have a lower risk of developing phaeochromocytoma compared to those patients with missense variants (58). The discovery of von Hippel–Lindau (VHL) syndrome and the VHL tumour suppressor gene, prompted a surge of interest in the study of rare inherited cancer syndromes as a model to gain insights
into the underlying biology of cancers but also to identify novel phaeochromocytoma and renal cell carcinoma predisposition genes, which affected similar molecular pathways.

Between the years 2000 and 2010, nine further phaeochromocytoma predisposition genes were identified and this era of discovery completely dispelled the previous belief in the ‘rule of ten’s’. This era of discovery was triggered by the seminal finding that families affected by a rare extra adrenal form of paraganglioma arising from the carotid body, carried a variant in the succinate dehydrogenase subunit D gene (*SDHD*)(59). The carotid body normally plays a crucial physiological role in the human body’s response to hypoxia and this prompted the hypothesis that the gene responsible for the development of these rare familial tumours must play a critical role in the oxygen-sensing and signaling pathways (59) and led to the subsequent investigation of the *SDHD* gene due its central role in the citric acid cycle and electron transport chain. Between 2000 and 2011, germline variants in all four of the succinate dehydrogenase subunit genes (*SDHA, SDHB, SDHC, and SDHD*) were implicated in the development of familial PPGL (60)(61)(62), as well as the *SDHAF2* gene which encodes its namesake protein responsible for the flavination of the SDHA protein (63).

In 2010, an integrated genomics approach led to the finding that a gene encoding a transmembrane protein (*TMEM127*) localised to chromosome 2, was implicated in familial PPGL(64). This transmembrane protein normally functions as a modulator of the mTOR pathway and notably patients with mutations in the *TMEM127* gene were found to present at an older age compared to patients with other syndromic causes of PPGL(64). One year later in 2011, aided by advances in genetic sequencing technologies, whole exome sequencing successfully identified recurrent variants in a novel gene called *MAX* or the MYC associated factor X gene in three unrelated patients with syndromic PPGL(65). Subsequent analysis suggested that this gene similar to *SDHD* and *SDHAF2* demonstrated a preferential paternal transmission pattern of inheritance (65).
Finally in 2013, a recurrent variant in another citric acid cycle enzyme encoding gene fumarate hydratase (FH) was implicated in hereditary phaeochromocytoma in addition to its previously accepted role in hereditary leiomyomatosis and renal cell cancer (66). PPGLs are now considered to be the most heritable of all tumours with 40% of patients having a genetic predisposition in one of 11 different susceptibility genes routinely screened for in clinical practice (SDHA, SDHB, SDHC, SDHD, SDHAF2, NF1, RET, TMEM127, MAX, FH, VHL). A number of additional predisposition genes have been reported in the past five years; such as the gene encoding the citric acid cycle enzyme malate dehydrogenase 2 (MDH2)(67) and the gene EPAS1 (68), which encodes the hypoxia inducible factor-2 alpha (HIF-2alpha). Both somatic gain of function variants (69) and germline variants in EPAS1(70) have been described in association with PPGL and pathogenic variants reported to date have resided in exon 9 and 12, which span HIFα hydroxylation sites (71). However, further studies are required to justify the introduction of routine screening for the MDH2 and EPAS1 genes into clinical practice.

1.12: The role of the succinate dehydrogenase (SDHx) genes in normal cellular function

The succinate dehydrogenase enzyme complex (SDH) is a key enzyme coupling the oxidation of succinate to fumarate in the citric acid cycle and the reduction of ubiquinone to ubiquinol in the electron transport chain. Therefore SDH facilitates the cellular metabolism of lipids, glucose and amino acids and feeds into the mitochondrial respiratory chain to generate cellular energy. The SDH complex is composed of four subunits, two hydrophilic subunits; SDHA and SDHB and two hydrophobic subunits SDHC and SDHD. The SDHA subunit is a flavoprotein and covalently binds a flavine adenine dinucleotide (FAD) co-factor and contains the binding site for the succinate metabolite, coupling the oxidation of succinate to
fumarate and reduction of FAD to FAD$_2$. The SDHB subunit contains three iron clusters which are employed in the transfer of electrons from FAD$_2$ to ubiquinone. Two LYR motifs are essential binding sites to enable the incorporation of the three Fe-S clusters within the final structure of complex II. The SDHC and SDHD subunits are embedded in the inner mitochondrial membrane and facilitate the binding of ubiquinone. Each subunit of the SDH complex is encoded by its namesake gene, the SDHA gene is located on the short arm of chromosome 5 (5p15), the SDHB gene at 1p36.13, SDHC at 1q23.3 and SDHD at 11q23.

1.13: Functional impact of SDHx mutations on the SDH complex

Most reported analysis for the functional and structural consequences of SDH gene variants, exist for SDHB gene variants (72).

The SDHB subunit forms the core of the SDH complex and a truncating variant in the SDHB gene will lead to degradation of the complex and absent SDH enzymatic activity. However, the majority of disease associated variants in the SDH gene complex are missense variants. Missense variants in SDHB are believed to cause functional impact by one of the following mechanisms; i) impaired stability of the protein (72), ii) impaired or reduced SDH enzymatic activity (72),(73), iii) impaired trafficking of the SDH complex to the mitochondria (72), iv) impaired co-assembly of the SDHA and SDHB subunits (affecting the catalytic site of the complex) (72), v) impaired incorporation of iron clusters into the SDHB subunit due to disrupted binding of LYR motifs(74)(75), (vi) disruption of normal splicing.
1.14: Pathways implicated in SDH related tumorigenesis

1.14 a): Hypoxia signaling

The first mechanism of tumourigenesis implicated in patients with pathogenic variants in the SDH complex was oxygen sensing and signaling because of the observation that $SDH_x$ variants predisposed to the development of carotid body tumours, which are also more commonly diagnosed in patients living at high altitudes exposed to chronic hypoxic conditions (76). The hypothesis is that $SDH_x$ variants activate hypoxia signaling pathways which are further stimulated by hypoxic environmental conditions at higher altitudes. This hypothesis is strengthened by a number of gene expression studies, which identified an upregulation of hypoxic transcription factors in $SDH$ mutated tumours (77), analogous to the transcriptomic profile of tumours with von Hippel Lindau gene ($VHL$) mutations (78). The VHL tumour suppressor gene is an important regulator of the hypoxia signaling pathway as it is responsible for the ubiquitin mediated degradation of the hypoxia inducible factor subunit alpha (HIFα) under normoxic conditions (77). Analysis of PPGL tumours with $SDH_x$ pathogenic variants revealed an upregulation of HIF targets (78), suggesting a molecular overlap in the transcription signature of tumours with associated $VHL$ and $SDH_x$ variants. Understanding the molecular background of patients with inherited phaeochromocytomas and paragangliomas enabled further interrogation of the associated transcription profiles and segregation of inherited PPGL into two categories (79). Category 1 is referred to as the ‘pseudohypoxic cluster’ based on the up-regulation of hypoxia signaling pathways and ‘cluster 2’ is characterised by an upregulation of kinase signaling pathways. Tumours caused by variants in the $VHL$ or $SDH_x$ genes fall into the pseudohypoxic cluster.
1.14 b): Succinate as an ‘oncometabolite’

Variants in the SDH complex genes result in an accumulation of the metabolite succinate as oxidative dehydrogenation of succinate to fumarate in the citric acid cycle is no longer feasible (80). The accumulation of succinate is postulated to be a key driver in tumourigenesis and succinate is now referred to as an ‘oncometabolite’ (81). The post translational regulation of HIFα subunit is a crucial regulatory step in the stabilisation of HIF. The hydroxylation of specific proline residue on HIFα by prolyl hydroxylases (PHD) destabilises HIFα, enabling the targeting of HIF for proteasome mediated degradation. The activity of the PHD enzymes is dependent on a number of co-factors including 2-oxoglutarate, iron and oxygen. Succinate is similar in structure to 2-oxoglutarate and the accumulation of succinate competitively inhibits 2-oxoglutarate and its associated dependent enzymes including PHD (82). Reduced activity of PHD destabilises HIFα and upregulates HIF and downstream HIF targets in the presence of a mutated SDH complex (77)(82).

1.14 c): Hypermethylation

Accumulation of the oncometabolite succinate is also responsible for the inhibition of other 2-oxoglutarate dependent dioxygenase enzymes including the JMJC histone demethylase class of enzymes and the ten eleven translocase (TET) DNA demethylase enzymes (83). These two enzyme classes are crucial regulators of genomic methylation and inhibition of these enzymes leads to a global hypermethylation pattern (83). The JMJC histone demethylase enzymes rely on 2-oxoglutarate to enable oxidation of the methyl groups in the side chains of the lysine and arginine amino acids of histone 3 and histone 4. Similarly, the TET enzymes are again dependent on the decarboxylation of 2-oxoglutarate to derive an iron oxide which can hydroxylate 5 methylcytosine (5mc) to 5 hydroxy methylcytosine (5hmc) (84). Therefore
in the presence of a defective SDH complex, elevated succinate will inhibit 2-oxyglutarate and cause dysregulated histone and DNA methylation and affect gene transcription.

SDH mutant PPGL tumour tissue, has demonstrated a global hypermethylation pattern as well as reduced expression of 5hmc on immunostaining(84), and in the same study, chromaffin cells extracted from a SDHB null mouse demonstrated the same hypermethylation phenotype observed in the SDH deficient tumour tissue. Furthermore administration of the demethylation agent decitabine successfully reversed the hypermethylation profile, highlighting the role of therapeutics in this reversible epigenetic phenomenon.

A recent study has also demonstrated a reduced expression of 5hmC and increased expression of the H3K9me3 methylation in SDH deficient tumour tissue and these findings were confirmed in vitro by SDHB knock down, implicating both histone and DNA hypermethylation in SDH deficient tumorigenesis(85). Furthermore this epigenetic alteration has also been identified in SDH deficient GIST tumours (86).
Figure 1.2: Discovery timescale of PPGL susceptibility genes

Figure 1.3: Illustration of the implicated pathways of tumorigenesis in SDH deficient disease. Adapted from (78)
1.15: Genotype-phenotype correlations

A review of $SDHx$ variants published by Evenpoel et al in August 2015, referred to a total of 445 germline pathogenic variants in the SDH complex, reported in the literature at that time. The majority of variants (403) were reported in association with PPGL (87). Missense variants were the most common variant type affecting $SDHA$, $SDHB$ and $SDHC$ gene subunits but frameshift variants were more commonly reported for $SDHD$ (87). $SDHx$ variants are heterozygous variants inherited in an autosomal dominant fashion and tumourigenesis is initiated by loss of the second (wild type) allele.

1.15 a): SDH deficient PPGL

A phaeochromocytoma is a tumour arising from the adrenal medulla and a paraganglioma refers to its extra adrenal counterpart, which can develop from sympathetic or parasympathetic tissue anywhere from the skull base to the pelvis. Paragangliomas in the abdomen and pelvis commonly arise from the aortic bifurcation or the so called ‘organ of Zuckerlandl’, but can also occur in the urinary bladder. Mediastinal PGL are rare but can develop from para-aortic (middle mediastinum) and para-vertebral (posterior mediastinum) sympathetic chain ganglia (88). Head and neck paragangliomas (HNPGL) can arise from the carotid body, glomus jugulare or glomus tympanicum and skull base (89).

The estimated incidence of PPGL is 3-5/million of the population per year, giving an annual prevalence of 192-320 new patients a year in the United Kingdom. Pathogenic variants in one of the four $SDHx$ genes account for 30-40% of hereditary PPGL (87), therefore we should expect 29-50 new patients with a diagnosis of SDH deficient PPGL per year in the United Kingdom. Variants in $SDHC$ and $SDHD$ were first associated with PPGL in 2000 (59)(62),
followed by the discovery that \textit{SDHB} was implicated in PPGL by Astuti et al\cite{60}. It was almost a decade later before a truncating germline variant in \textit{SDHA} was identified in a patient with PPGL\cite{61}. The majority of germline \textit{SDH} variants reported to date in association with PPGL, have been identified in the \textit{SDHB} gene (52%), followed by variants in \textit{SDHD} (35\%)\cite{87}. Recently published Endocrine Society guidelines on the management of phaeochromocytoma and paraganglioma have recommended that genetic screening should be considered in all patients with PPGL\cite{90} and that \textit{SDHx} variants in particular should be screened for in patients with extra adrenal PGL\cite{90}.

Phaeochromocytomas and abdominal PGL are frequently secretory and patients can present with symptoms of catecholamine excess including hypertension, palpitations, anxiety and headaches. Head and neck PGL are less commonly secretory and therefore, patients may present with compressive symptoms such as tinnitus, cranial nerve palsies, hearing loss or dysphagia. Recently, as genetic testing for patients with PPGL has increased, PPGL are being diagnosed by radiological and biochemical surveillance of \textit{SDHx} carriers, picked up by family screening.

The strongest existing genotype-phenotype correlation for \textit{SDHx} mutated PPGL, is the risk of malignancy associated with \textit{SDHB} mutated PPGL. Pathogenic \textit{SDHB} gene variants confer an estimated 50\% risk of malignant transformation\cite{5} and a five year survival of less than 50\% if malignant disease does occur. Extra adrenal paragangliomas in the abdomen and pelvis as well as adrenal phaeochromocytomas are most commonly associated with pathogenic variants in the SDHB subunit compared to the other subunits, but tumours can arise in the head and neck and thorax.\cite{5}\cite{91}\cite{92}.

Variants in the \textit{SDHC} and \textit{SDHD} genes are most commonly associated with head and neck PGL and rarely associated with thoraco-abdominal PGL or PC. \textit{SDHD} is the most common
SDHx gene implicated in HNPGL and tumours are frequently multifocal with a lower malignant potential compared to SDHB (93).

Until 2010, when the first association was made between PPGL and the SDHA subunit51, SDHA gene variants were associated only with autosomal recessive metabolic encephalopathy syndrome (Leigh syndrome). SDHA variants are still considered a rare hereditary cause for PPGL, thought to be related to the low frequency of 5p15 (SDHA gene locus) loss seen in PCC and PGL (61).

1.15 b): SDH deficient GIST

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumours of the gastrointestinal tract. Most GISTs occurring in adults are driven by activating mutations in the KIT(94) or PDGFRA(95) genes. GIST that harbour KIT or PDGFRA mutations have lower malignant potential and an excellent response to the tyrosine kinase inhibitor Imatinib. Wild type gastrointestinal stromal tumours (GIST), refer to those tumours that are negative for KIT and PDGFRA somatic gene variants (96)(97) and account for 15% of adult and 85% of paediatric GIST tumours. Data from a recent observational study suggest that approximately 88% of wt GIST are caused by a pathogenic germline variant in the SDH complex(97).

A subsequent report from the NIH, confirmed that SDH deficiency accounted for 88% of wt GIST in their cohort(98). Of this 88%, the majority of cases had an identified germline SDHx variant (75%) and the remainder (25%) had an epimutation in the SDHC gene, caused by hypermethylation of the promoter region of the SDHC gene(98).

A strong genotype-phenotype correlation exists for SDHA germline variants and SDH deficient GIST, as pathogenic SDHA variants account for 47% of cases(87). Missense variants in the SDHx genes are the most common type of variant reported in SDH deficient
GIST. To date 16 different germline $SDHx$ variants have been reported in patients with both GIST and PPGL(87).

Unlike conventional GIST tumours, which have a female preponderance, the female to male ratio is equal in SDH deficient GIST and the age of presentation is often later than conventional GIST, with a number of reported cases presenting over the age of 50 years(97)(98). SDH deficient GIST have only been reported in the stomach with only one cases of small bowel SDH deficient GIST to date(99). The primary tumour is often multifocal along the stomach wall and tumours have a characteristic histological epithelioid appearance(99).

SDH deficient GIST tumours are challenging from a management standpoint as they do not respond to standard therapy with Imatinib (98)(100) and have higher tendency to metastasise. (97)(98)(100). One of the largest observational studies of wt GIST to date, reported that 32% of patients with wt GIST have metastases at presentation and 60-80% of all patients will develop distant metastatic disease, which then runs an indolent but unpredictable course(100). Syndromic wt GIST refers to the occurrence of Carney-Stratakis syndrome due to a germline mutation in SDHx or Carneys triad, which occurs exclusively in patients with a somatic epimutation in the $SDHC$ gene, rather than an inherited germline $SDH$ mutation (98)(101)(102) Carney’s triad refers to tumour syndrome consisting of pulmonary chondroma tumours, paraganglioma and wt GIST. Initially, a large somatic genomic deletion on 1q, encompassing the $SDHC$ gene locus, was postulated as the molecular driver of this tumour syndrome(101). However additional studies did not replicate this finding and a study by Killian et al in 2014 confirmed that an epimutation of the $SDHC$ gene due to
hypermethylation of the promoter region of this gene, rather than a sequence mutations was implicated in Carney’s triad (102).
Figure 1.4: Schematic illustration depicting the molecular subtypes of wild-type gastrointestinal stromal tumours (GIST).

![Diagram showing molecular subtypes of GIST]

Figure 1.5: Displays the two most common gene predisposition syndromes (SDHx and NF1) associated with wild-type GIST. This diagram highlights the differences in clinical presentation and histology between SDH deficient and SDH preserved wild-type GIST syndromes.

![Diagram showing clinical presentation and histology differences]

1. SDH deficient WT GIST
2. SDH preserved WT GIST
1.15 c): SDH deficient renal cell carcinoma (RCC)

Fifteen years ago, following the description of pathogenic germline SDHB variants as a cause of phaeochromocytoma, paraganglioma and HNPGL, a SDHB variant was detected in two kindreds with PPGL and RCC(3). SDH deficient RCC account for approximately 0.2% of all cases of RCC(103) and to date 83% of the germline variants identified in SDH deficient RCCs occurred in SDHB(87). Patients with a germline SDHB variant have an estimated 14% life time risk of developing an SDH deficient RCC(93)and the mean age at presentation is approximately 35-40 years(103)(104). Germline variants in the other three subunits; SDHA(105), SDHC(106), SDHD(107), have also been associated with hereditary RCC. Notably, SDH deficient RCC has been reported in patients with apparently sporadic RCC with no family history of RCC or PPGL(108). The co-occurrence of other SDH deficient tumours such as PPGL and GIST was observed in 9 patients or 30% in one cohort study(103).

SDHB-deficient renal carcinoma has recently been accepted as a provisional entity in the 2013 ISUP Vancouver Classification and a unique morphology consisting of solid architecture, intracytoplasmic inclusions and intra tumour mast cells is characteristic of SDHB deficient RCC(109) The morphological appearance of SDHB deficient RCC is so distinct that it is recommended that molecular genetic testing be carried out when it is encountered(109). Metastatic disease has been reported in SDH deficient RCC and is more common in patients with a high histological grade, specific histological features such as coagulative necrosis or tumours with features of de-differentiation (103)(110).

1.15 d) : SDH deficient pituitary adenomas (PA)

To date, there have been eight reported cases of SDH deficient pituitary adenomas and the estimated incidence is 0.3% (111). Three of the eight reported cases were found to have a
germline variant in SDHD, two cases each of SDHB and SDHC mutated PA and one patient had a pathogenic variant in SDHA(87)(112). The cause and effect relationship between PA and SDHx gene variants requires further investigation, owing largely to the limited functional assessment of tumour tissue, as prolactin secreting pituitary adenomas are predominately treated by medical management and non-functioning adenomas only require surgical resection if compressive symptoms develop.

Based on limited and predominately anecdotal observations, SDH deficient pituitary adenomas are more commonly macro-adenomas, present at a younger age, and are either non-functioning or prolactin secreting, with a slightly higher predisposition among male patients. A characteristic finding of intracytoplasmic vacuoles on histology review, has also been demonstrated in SDH deficient pituitary adenomas in one study(4)(112).

1.15 e): Rare SDH deficient tumours

Twenty three different germline variants in SDHx have been identified in patients with atypical tumours(87) including; breast, thyroid carcinoma, testicular seminoma and neuroblastoma. The direct causal role of SDH deficiency in this tumours is difficult to determine based on existing reports, because of a relative lack of reported functional analysis.

Differentiated thyroid cancer (DTC) including follicular and papillary thyroid cancer have been reported in carriers of SDHx variants. Papathomas et al performed SDHB immunohistochemistry on 60 papillary thyroid carcinoma (PTC) samples from patients with unknown SDHx germline mutational status and one case of PTC from a patient with a pathogenic SDHD variant. No case of SDHB immunonegativity to suggest an SDH deficient PTC was detected and retention of the wild type allele was observed in the patient with the known germline SDHD variant(113). Given the high prevalence rates of PTC (7.7%), it is
possible that PTC is being incidentally discovered in SDHx carriers undergoing frequent radiological surveillance of the neck.

Another hypothesis is that there is pathway signal interaction between the tumour suppressor gene phosphatase and tensin homolog (PTEN) and germline SDHx variants, which lead to Cowden syndrome like tumourigenesis(114)(115). This is based on the observation that the co-occurrence of germline variants in SDHB/C/D (SDHx) and germline PTEN variants, conferred a higher risk of breast and thyroid cancers, over those with only germline PTEN variants alone. At a tissue level, the highest level of reactive oxygen species was in the tumours from patients with both PTEN and SDHx germline variants, compared to those with a single variant in either PTEN or SDHx. The increased level of reactive oxygen species was associated with increased resistance to apoptosis and P53 degradation in the SDHx germline variant carriers (114). However, the studies performed to date have not confirmed SDH deficiency in PTC (114)(115).

A novel association between a germline SDHD (c.129G>A, p.Trp43X) variant and a testicular seminoma has been confirmed in a single patient, by demonstrating loss of the wild type allele in the tumour tissue (116).

To date, there has been no reported case of an individual with a germline SDHx variant causing the co-occurrence of the three more common associated tumour types of PPGL, GIST and RCC. The incomplete penetrance of ‘SDH syndrome’ may be explained by differences in tissue specific requirements for ATP and the metabolic intermediates produced by the TCA cycle, altering the threshold for neoplastic transformation due to complex II dysregulation in different tissues (117).
1.16: Disease penetrance with pathogenic germline SDHx variants

SDHx variants are heterozygous variants inherited in an autosomal dominant fashion with an age dependent and incomplete penetrance. As per Knudson’s theory of tumourigenesis, loss of the second wild type allele must occur for tumourigenesis to be initiated in patients with a germline SDHx variant. Similar to the other PPGL predisposition genes identified to date, SDHA, SDHB and SDHC are inherited in an autosomal dominant pattern. An additional inheritance mode has also been identified for SDHD, SDHAF2 and the MAX gene, namely a parent of origin effect, where disease penetrance is almost exclusively dependent on paternal transmission (118), although rare reports of disease penetrance with maternal transmission exist for SDHD (119). The initial hypothesis that a paternally inherited SDHD/SDHAF2 variant together with a maternally imprinted silenced wild type allele, has been replaced by an alternative hypothesis, namely the ‘Hensen model’ of inheritance (120). The ‘Hensen model’ suggests that the somatic loss of the maternal copy of chromosome 11 in the tumour of individuals with a paternally inherited SDHD/SDHAF2 variant, results in loss of wild type allele function at this locus but also a maternally expressed, paternally imprinted gene in the 11p15 region, as this region contains an important cluster of imprinted genes (120). This model is further supported by the observation that many VHL related PPGL also exhibit loss of 11p (121).

Initially the penetrance of SDHB variants was estimated at approximately 70-80% (122), but as genetic screening for asymptomatic relatives has become more common place, more recent studies suggest a penetrance of <50% in non-probands (123). The penetrance in this study and others, adopted standard Kaplan Meir statistical methods, which does not account for ascertainment bias and can overestimate risk. A study by Schiavi et al, applied a modified segregation analysis to a cohort of 135 SDHB variant carriers and estimated an average penetrance of 13% by age 50 years and an overall average lifetime risk of 30% (124).
The incomplete and age dependent penetrance observed in patients with germline $SDHx$ variants is likely multifactorial, owing in part to the previously discussed tissue specific thresholds for neoplastic transformation. Another possible explanation, is the variation that has been observed in the mechanisms by which biallelic inactivation occurs. Loss of the second allele has been reported to occur by loss of heterozygosity(61), hypermethylation or epigenetic silencing of the second allele and by a co-occurring somatic mutation(125). There is no evidence at present to suggest that $SDHx$ related tumourigenesis is related to haploinsufficiency(117).

1.17: The role of next generation sequencing in the detection of $SDHx$ variants

Next generation sequencing (NGS) has facilitated a cost effective and time efficient transition from previous methods of targeted, sequential, analysis of individual susceptibility genes in hereditary PPGL(126)(127). NGS can be performed on both germline DNA and DNA extracted from fresh frozen or paraffin embedded tumour samples. It has been estimated that a second-generation sequencing test for a ‘panel’ of ten susceptibility genes predisposing to phaeochromocytoma ($MAX$, $RET$, $SDHA$, $SDHB$, $SDHC$, $SDHD$, $SDHAF2$, $TMEM127$, and $VHL$), can achieve a 70% cost reduction compared to standard targeted sequencing methods of individual candidate genes(126). More recent NGS panels also include the most recent gene implicated in hereditary PPGL; fumarate hydratase ($FH$)(66). The sensitivity of multiple parallel sequencing is not compromised, with centres achieving a diagnostic sensitivity of 98.7%(126). Many centres however, continue to confirm or validate mutations detected on NGS by Sanger sequencing. A potential pitfall of NGS techniques, is the limited (albeit improving) ability of this technique to detect exon deletion/duplication mutations. Exonic deletion mutations account for approximately 10% of reported $SDHx$ mutations(87).
Techniques such as multiple ligation-dependent probe amplification and multiplex PCR can be applied in the detection of these large deletion mutations(128)(129).

One of the main drawbacks of NGS is the frequency at which variants of uncertain significance are encountered. SDHx variants of uncertain significance are frequently encountered, and in one study validating a comprehensive NGS genetic strategy for hereditary PPGL, 15/36 (41.6%) novel variants detected were in SDHx genes(126). These variants cannot always be interpreted readily with existing and accessible bioinformatics or functional techniques. Therefore the clinical significance of certain SDHx variants diagnosed by NGS, may rely on the collation of further sequencing and phenotypic data, thus creating uncertainty for patients and physicians.

1.18: Surveillance protocols for pathogenic SDHx variant carriers

It is recommended that asymptomatic carriers of pathogenic SDHx variants undergo regular surveillance in order to facilitate early detection of SDHx associated tumours and reduce the morbidity associated with these undiagnosed tumours (90). This surveillance is advised to begin at the age of 11 years and continues life-long, if acceptable to the patient(130).

Although regular surveillance for pathogenic SDHx variant carriers is a widely accepted practice, there is no consensus on the frequency of surveillance or on the optimal radiological modality. The current recommendation is that annual clinical review is performed in addition to measurement of plasma metanephrines or urine metanephrines (90). However the frequency of cross sectional imaging and the choice of radiological modality differs between practices and although the Endocrine Society guidelines recommend cross sectional imaging for SDHx carriers, to diagnosis non-secretory tumors, they do not specify appropriate time intervals between imaging studies or a preferred imaging modality(90). In recent years, as
updated information regarding disease penetrance of the pathogenic SDHx variants has become available, concern regarding unnecessary radiation exposure in this group has mounted and more centers are recommending MRI alone as the imaging modality of choice(130). This approach is supported by a recent study which identified that MRI was as sensitive as $^{18}$F-fluorodeoxyglucose (FDG) PET CT in the diagnosis of occult tumours in SDHx carriers (131). Finally the interval frequency at which radiological screening is carried out remains contentious but it is widely accepted that the first radiological screen is the most important and yields the highest diagnoses of tumours in this cohort(130)(131). At present, studies would support a two yearly interval between radiological screens but there is a need for additional long-term surveillance data to better inform this challenging area of practice.
Figure 1.6: Demonstrates the accepted clinical, biochemical and radiological surveillance strategy for SDHx carriers (90)
1.19 Treatment options for metastatic SDH deficient disease

Surgery remains the only chance of cure for patients with SDH deficient tumors. The lifetime risk of malignant PPGL with pathogenic SDHx variants is approximately 10-20%, with the highest risk observed in patients with germline pathogenic SDHB variants. The five year survival if malignant disease develops is less than 50%(5). After surgical resection, effective treatment options are limited and palliation is often the only noted benefit of these treatment options(132). There is a need for further development of targeted therapies in order to improve progression free survival rates for patients with metastatic SDH deficient disease.

1.19 a): Treatment options for metastatic PPGL

Treatment options for metastatic PPGL are aimed at controlling tumour proliferation but also tumour secretion of catecholamine's in order to reduce the morbidity associated with excess catecholamine circulation. Systemic treatments include cytotoxic chemotherapy consisting of a regime of cyclophosphamide, vincristine and dacarbazine (CVD). A meta-analysis deemed that this regime was associated with disappointing rates of progression free survival and low rates of complete and partial disease remission(133). However a study by Fishbein and colleagues in 2017, demonstrated that SDHB carriers had a better response to CVD chemotherapy compared to patients without a pathogenic SDHx variant (134). This study was a retrospective study of a small cohort of 23 patients and therefore larger prospective studies are needed to confirm this finding.

Other systemic treatment options include peptide receptor radionuclide therapy (PRRT) with radio-labelled somatostatin analogues and (131) I-metaiodobenzylguanidine (MIBG) therapy. A meta-analysis published in 2014 investigating the effect of MIBG therapy on complete and partial disease response in malignant PPGL, demonstrated that a partial response was
achieved in 40%(135), however MIBG therapy may have a less significant role in the treatment of SDH deficient PPGL as patients with pathogenic SDHx variants have reduced avidity on MIBG scintigraphy(136), predicting a poor response to MIBG therapy. A recent study demonstrated that PRRT treatment using (90) YDOTATATE was comparable to MIBG therapy in terms of rates of overall survival and progression free survival achieved(137).

Advances in pan-genomic analysis has promoted a targeted approach to the new development of molecular therapies in SDH deficient tumours and agents such as sunitinib (138) and temozolomide(139) have shown promise as effective targeted therapies in the treatment of SDH deficient PPGL but larger prospective studies are needed to confirm these results.

1.19 b): Treatment of SDH deficient GIST

Evidence supporting the use of targeted therapies in SDH deficient GIST is limited. Data from one of the largest observational studies of adult wt GIST patients to date, identified a poor objective response to tyrosine kinase inhibitors, but data was collected from medical records and was not standardised (98). Smaller studies in pediatric patients with wt GIST have demonstrated a good response to sunitinib therapy following disease progression on imatinib, but SDHx mutational status of these patients is not known(140). In the future, advances in the molecular classification of wt GIST may lead to a more informed selection of therapies for individual patients such as de-methylation agents for patients with a diagnosis of an SDHC epimutation.
1.19 c): Treatment of other SDH deficient tumours

Evidence for the treatment of SDH deficient RCC and pituitary carcinoma is limited to anecdotal cases; including the use of temozolomide in a patient with pituitary carcinoma, who benefited in terms of clinical response and disease stability (141), and the use of pazopabnib in a patient with metastatic SDH deficient RCC, who despite initial partial response to this treatment, died within 19 months of presentation (142).

1.2 Barriers to optimal clinical practice

Despite recent advances in our understanding of the SDHx genes, including improved recognition of their molecular signatures, penetrance patterns and genotype-phenotype correlations, there are existing areas of unmet clinical need and a number of barriers to optimal clinical practice still exist.

1.21: Interpretation of SDHx variants of uncertain significance

Functional assessment is particularly important for the interpretation of SDHx variant pathogenicity as the majority of disease associated variants are missense and therefore the validation of new sensitive and accessible techniques are required, to ensure correct interpretation of novel SDHx variants, to facilitate appropriate clinical surveillance and family screening.
1.22: Limited biomarkers to predict and monitor malignant disease

Accepted clinical predictors of malignant potential in patients presenting with PPGL tumours include; large tumour size (>4cm), extra adrenal abdominal location, and a pathogenic germline SDHB variant (143)(144). Histological predictors of the malignant potential of PPGL include a poorly differentiated tumour morphology and loss of SDHB protein expression on immunohistochemistry testing, however other indices such as the KI67 proliferation index, are less specific and no longer accepted as a single indicator of malignant potential in this tumour group (144). Biochemical biomarkers are limited but plasma 3-methoxytyramine, an O-methylated derivative of dopamine, was recently evaluated as a potential biomarker of malignant disease(145), however sensitivity and specificity of this biomarker were suboptimal (sensitivity 57% and specificity 85%)(145).

Unfortunately, robust biomarkers for prediction of malignant risk with GIST are lacking. However, it is well accepted that SDH deficient wt GIST have higher rates of metastases and local recurrence post-surgery and therefore it is now recommended that SDHx status of wt GIST is confirmed early in the diagnosis, particularly for patients presenting under the age of 30 years with gastric multifocal tumours, as the risk of SDH deficiency is highest in patients with this phenotype(98)(146). Therefore, despite advances in our knowledge of the molecular signature associated with SDH deficient tumours, there are limited robust biomarkers to predict malignant potential and tumour recurrence (132). Furthermore, identification of metastatic spread remains heavily dependent on the use of functional imaging studies, some of which are less sensitive in patients with pathogenic SDHx variants (136). Importantly evaluation of sensitive translational surrogates of response would guide the use of existing personalised treatment options as well as aid the development of new effective treatments.
1.23: Study hypothesis

Study hypotheses included

i) A multi-omics approach to the study of SDH deficient tumours (including targeted analysis of the tumour genome, metabolome and methylome), may reveal new biomarkers or therapeutic targets

ii) Current clinical surveillance practices may be better informed by expanding on existing genotype phenotype studies
1.24 Aims of PhD study

The aim of this PhD study is to study the mechanism of tumorigenesis implicated in SDH deficient disease in order to:

1. Identify new genotype-phenotype correlation, which may inform better surveillance practices

2. Evaluate new functional tools which could aid the clinical interpretation of SDHx variants of uncertain significance

3. Identify new translational biomarkers, which could be used for diagnosis, surveillance and to monitor response to therapeutic intervention in SDH deficient disease.

4. Investigate new potential targets for therapeutic development
Chapter 2

General Methods
Chapter 2: General methods

2.1 Nucleic acid extraction

2.12 DNA extraction from blood

2.13 DNA extraction from fresh frozen tumour

2.14 DNA extraction from paraffin embedded tissue

2.15 RNA extraction from paraffin embedded tissue

2.2 cDNA synthesis

2.3 Sanger sequencing

2.31 Primer design

2.32 PCR for Sanger sequencing

2.33 Sequencing clean up

2.34 Sequencing

2.35 Sequencing analysis

2.4 Next generation sequencing

2.41 Library preparation

2.42 Sequencing methods

2.5 Bioinformatics analysis

2.6 Germline genetic sequencing analysis of clinical samples
2.1 Nucleic acid extraction

2.1.2 DNA extraction from blood

Germline DNA was extracted by colleagues at the Clinical Genetics Laboratory, Cambridge University Hospital NHS Foundation Trust. DNA was extracted from lymphocytes using the Gentra Puregene Blood Kit (Qiagen, UK) according to the manufacturer’s instructions and subsequently quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

2.1.3 DNA extraction from fresh frozen tumours

DNA extraction from 10-20mg of fresh frozen fresh frozen tumour was performed by the staff at the Stratified Medicine Core Laboratory within the Academic Department of Medical Genetics, Cambridge University. The pre-extraction protocol was as follows; tissue samples were transferred to a 2ml micro centrifuge tube and 220µl Buffer ATL was added followed by 20µl of proteinase K and the sample was mixed. The tube was transferred to ThermoMixer and incubated at 56°C, at 900 rpm until the tissue was completely lysed and average lysis time was three hours. Following tissue lysis, 4µl RNase A (100mg/ml) was added and the sample was incubated for 2 minutes at room temperature. The sample was homogenised by pipetting and if necessary centrifuged at 3000 x g for 1 minute. Finally 200µl of the supernatant was transferred to labelled sample tubes. Extraction was then performed using the Qiasymphony automated DNA extraction protocol (Quiagen, UK).
2.14: DNA extraction from paraffin embedded tumour samples

Tumour microdissection was performed by colleagues in the Department of Haematology and Oncology diagnostic services, Cambridge University Hospital. Tissue slides were de-paraffinised by two soakings for 10 min in 100% xylene, and two soakings for 10 min in 100% ethanol and then air-dried. After histological identification and selection of the tissue region to be processed, the relevant tissue was scraped off using a sterile scalpel blade (Swann Morton No.11) into 80 microliter ATL (Qiagen 56404). 20 microliter proteinase K was added and the tissue was incubated for 3 days at 56°C with a daily 10 microliter proteinase K supplement. Subsequently, the digested tissue was incubated for one hour at 90°C, centrifuged, supplemented with 200 microliter of AL buffer, mixed by vortexing, supplemented with 200 microliter of 100% ethanol and mixed by vortexing and "briefly centrifuged. The lysate was loaded on a QiaAmp MinElute Column and centrifuged 10000 rcf for 1 minute, washed with 500 microliter of AW1, centrifuged at 10000 rcf for 1 minute, washed with 500 microliter AW2 and centrifuged at 16000 rcf for 3 min, then centrifuged dry for 3 min at 16000 rcf, and eluted with 50 microliter ATE buffer by incubation at room temperature for 5 min and centrifugation for 2 min at 10000 rcf.

2.15: RNA extraction from paraffin embedded tumour samples

RNA extraction was performed by Dr Rogier ten Hoopen (Department of Oncology, Cambridge University). RNA extraction slides were processed with the RNAstorm kit from CellDataSci (CD501) according to the manufacturer’s protocol. After histological identification and selection of the tissue region to be processed for RNA extraction the relevant tissue was scraped off using a sterile scalpel blade (Swann Morton No.11) into 500
microliter of de-paraffinisation reagent, vortexed for 10 sec and incubated for 3 min at 72°C, then allowed to cool to room temperature. 80 microliter of CAT5 reagent was added, vortexed for 10 sec and centrifuged for 1 min at 16000 rcf. Most of the clear yellow organic upper phase was removed and discarded, the remainder incubated for 30 min at 72°C, followed by adding 80 microliter lysis buffer and 10 microliter of protease K and continued incubation at 72°C for 2 hours. The RNA extraction was cooled for 3 min on ice and centrifuged for 15 min at 16000 rcf. The lower aqueous phase was removed to a new microcentrifuge container, supplemented with 150 microliter binding buffer, 450 microliter 100% Ethanol and mixed by inverting several times and loaded on a spin column. The spin column was centrifuged for 1 min at 16000 rcf and washed with 300 microliter of DNAse treatment wash buffer (1:1:3 of water, binding buffer and ethanol, respectively) and centrifuged for 1 min at 16000 rcf. 70 microliter Dnase I buffer with 2 microliter reconstituted DNase I was added to the spin column membrane, incubated for 15 min at RT and centrifuged for 1 min at 16000 rcf. Subsequently the spin column was washed with 300 microliter of DNAse treatment wash buffer and centrifuged for 1 min at 16000 rcf, 500 microliter Wash buffer, centrifuged for 1 min at 16000 rcf, and extra wash of 500 microliter Wash buffer, centrifuged for 1 min at 16000 rcf, and spun dry for 5 min at 16000 rcf. RNA was eluted from the spin column with 30 microliter RNAse-free water for 1 min incubation at RT and centrifugation for 2 min at 10000 rcf. RNA yield was measured on the Nanodrop ND-1000 Spectrophotometer and stored at minus 80°C.
2.2 cDNA synthesis

cDNA synthesis was performed by Dr Rogier ten Hoopen. Synthesis was performed with the Superscript III First–Strand Synthesis Supermix (Invitrogen 18080-400).

2.3 Sanger sequencing

2.31 Primer design

Primer design was performed independently. Primers were designed to validate by Sanger sequencing, those somatic variants that were identified by next generation sequencing and interpreted as being potential pathogenic driver variants. Primers were designed for the individual variants with the aid of the open access software programme Genome Compiler (http://www.genomecompiler.com). A full list of the designed primers employed are displayed in chapter 5.

2.32 PCR clean-up for Sanger sequencing

This was performed by Philip Smith in the Academic Department of Medical Genetics, Cambridge University. The Applied Biosystems AmpliTaq Gold PCR (Thermo Fisher UK) protocol was adapted. The PCR reaction was made up as outlined in Table 2.1. The thermal cycling conditions were as shown in Table 2.2.
Table 2.1 PCR Mastermix reagents and volumes

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (UL)</th>
<th>Concentration in Mastermix</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer II or 10X PCR Gold Buffer</td>
<td>5</td>
<td>1X</td>
</tr>
<tr>
<td>25 mM MgCl2 Solution</td>
<td>2-8</td>
<td>1.0-4.0 mM</td>
</tr>
<tr>
<td>dATP 10 mM</td>
<td>1</td>
<td>200µm</td>
</tr>
<tr>
<td>dCTP each</td>
<td>1</td>
<td>200µm</td>
</tr>
<tr>
<td>dGTP</td>
<td>1</td>
<td>200µm</td>
</tr>
<tr>
<td>dTTP</td>
<td>1</td>
<td>200µm</td>
</tr>
<tr>
<td>Forward Primer 1</td>
<td>1-5</td>
<td>0.2-1µm</td>
</tr>
<tr>
<td>Reverse Primer 2</td>
<td>1-5</td>
<td>0.2-1µm</td>
</tr>
<tr>
<td>Sample DNA</td>
<td></td>
<td>&lt;1µg</td>
</tr>
<tr>
<td>AmpliTaq Gold</td>
<td>0.25 units</td>
<td>1.25 units/reaction</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50μl</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2: Thermal cycling conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial Denaturation</td>
<td>95 degrees</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95 degrees</td>
<td>1 minute</td>
<td>10</td>
</tr>
<tr>
<td>3. Primer annealing</td>
<td>65 degrees</td>
<td>1 minute</td>
<td>10 (-1 degree Celsius each cycle)</td>
</tr>
<tr>
<td>4. Extension</td>
<td>72 degrees</td>
<td>1 minute</td>
<td>10</td>
</tr>
<tr>
<td>5. Denaturation</td>
<td>95 degrees</td>
<td>1 minute</td>
<td>20</td>
</tr>
<tr>
<td>6. Primer annealing</td>
<td>55 degrees</td>
<td>1 minute</td>
<td>20</td>
</tr>
<tr>
<td>7. Extension</td>
<td>72 degrees</td>
<td>1 minute</td>
<td>20</td>
</tr>
<tr>
<td>8. Final Extension</td>
<td>72 degrees</td>
<td>10 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>
2.33 Post PCR

An ExoSap method was used for post PCR clean up. An ExoSap ratio of 1µl Exonuclease I (Exo) (New England Biolabs, M0293L) to 2µl Shrimp Alkaline Phosphatase (SAP) (GE Healthcare, GZE70092Z) was used and 1ul of ExoSap was transferred to the PCR products and incubated at 37 °C for 60mins, followed by a deactivating stage @ 80°C for 15mins. Products of ExoSAP with BigDye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystem, 4337456) was used. For each sample, 10µl reaction was made up: 2µl purified PCR product, 0.75µl BigDye V3.1, 1µl desired (forward or reverse at 10pmol) primer, 2µl 5x BigDye sequencing buffer (CTS) + 4.25µl H₂O. The 10ul reaction sample was then placed on tetrad (MJ Research DNA Engine Tetrad PTC-225) for the following cycle sequence;

- 25 Cycles;
  96°C for 10secs
  50°C for 5secs
  60°C for 3mins 30secs

Unincorporated dyes were removed using isopropanol. 40µl of 75% isopropanol was added to each BigDye termination reaction well and mixed gently by pipetting up and down twice. The mixture was centrifuged at 2092 RCF for 45mins and hen inverted on to absorbent paper to remove the isopropanol supernatant and then placed inverted into the rotor bucket and centrifuged again at 33 RCF for 30secs. The plate was then left to air dry in the dark for 10 minutes and the pelleted DNA was then suspended in 10µl of Hi-Di™ Formamide (Applied Biosystems, cat no: 4311320) and 10µl of H₂O.

2.34 Sequencing

The reaction was then loaded on ABI 3730 analyser (Applied Biosystems).
2.35 Sequencing analysis

Sequencing data was then analysed using Mutation Surveyor (SoftGenetics) coupled with the appropriate gene specific reference file downloaded from UCSC Genome Browser Home (UCSC Genome Browser Home). Mutation Surveyor aids sequence analysis by comparing of the inputted electropherogram with the reference file and identifying discrepancies.

2.4 Next generation sequencing

Library preparation

i) Pre PCR:

Library preparation was performed by the staff at the Stratified Medicine Core Laboratory, using an Ampliseq on MiSeq protocol adapted from a NEBNext Ultra II protocol from Illumina (https://www.illumina.com). A two primer approach was used (P1 (orange) and P2 (blue)). Pre PCR, all samples were vortexed and spun down. A volume of each sample needed for a final concentration of 10ng in a total volume of 5ul was calculated and 18M water was used to make up the difference. Samples were then transferred to an individual well in a fortitude plate. Two separate dilutions for each sample was made up and the samples for primer 1 were placed in row A and B and then in the same sample orientation samples were placed in rows E and F for the second primer.

ii) PCR:

10ul of the appropriate primer was added to each sample (P1 to samples in rows A and B and P2 to samples in rows E and F). 25 ul of the Master Mix was then added to each sample and mixed, followed by an addition of 10 ul of 18M water to each sample. The plate was then heat sealed and pulse spun followed by thermal cycling according to the conditions outlined in Table 2.3
Table 2.3: PCR thermal cycling protocol

<table>
<thead>
<tr>
<th>Cycling Conditions</th>
<th>Reaction Mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>98 °C – 30 Seconds</td>
<td>Q5 Master Mix</td>
<td>25 µl</td>
</tr>
<tr>
<td>98 °C – 10 Seconds</td>
<td>Primer Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>60 °C – 30 Seconds x 3</td>
<td>DNA</td>
<td>5 µl</td>
</tr>
<tr>
<td>65 °C – 2 Minutes</td>
<td>Water</td>
<td>10 µl</td>
</tr>
<tr>
<td>65°C – 5 Minutes</td>
<td>Total Volume</td>
<td>50 µl</td>
</tr>
<tr>
<td>4°C - Hold</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

iii) Post PCR pooling and clean up:
Post PCR 3ul of NEB USER Enzyme was added to each well and the plate was heat sealed again, vortexed and pulse spun. The plate was then placed on the Eppendorf thermal cycler for fifteen minutes. Both primer 1 (53ul) and primer 2 for each sample (eg A1 with E1, A2 with E2) were combined and mixed by pipetting. 95.4 ul of thawed Ampure beads (x1.8) were transferred into a MIDI plate and mixed with 53ul of each sample. The combined mixture of beads and the sample were shaken for one minute at 1800rpm, incubated at room temperature for five minutes and then incubate at room temperature for 5 minutes and place on a magnet for 2 minutes or until the beads have cleared. The supernatant was extracted without disturbing the beads and 200ul of 80% ethanol was added to each sample and then removed again after 30 seconds. The ethanol washes were repeated two times and beads were then left to dry. 52ul of resuspension buffer was then added to each sample, shaken for 1 minute at 1800rpm and incubated for 2 minutes at room temperature and finally placed back onto the magnet for 2 minutes or until the beads cleared. 50ul of each sample from the MIDI plate was then transferred on into a new labelled fortitude plate and placed in the freezer.

iv) Ligation and adenylation:
7ul of the NEB End Repair reaction buffer was added to each sample using a new tip each time; followed by 3ul of NEB End Repair enzyme mix. Samples were mixed, spun and
placed on Eppendorf thermal cycler for one hour. 30ul NEB ligation master mix and 1ul of ligation enhancer was then added to each sample, followed by 2ul of the correct barcode to each sample. The barcode for each sample was carefully recorded. Samples were again mixed and placed on thermal cycler for 15 minutes.

v) **Ampure clean up:**
The Ampure bead clean up described earlier was repeated using 70.4 and then 30.8ul of ampure beads with two ethanol washes between each ampure bead clean up.

vi) **Quality Control steps:**
Libraries were quantified by qPCR following Kapa protocol and then a 1/1000 dilution of each sample was made by transferring 2ul of final library in 38ul of water, take 2ul from this dilution in 98ul of water.

vii) **Sequencing on the MiSeq:**
5ul from each sample was transferred into a 1.5ml tube. The appropriate MiSeq protocol and the correct parameters to sequence the 2nM library was followed.
2.5 Bioinformatics analysis

The bioinformatics analysis of research samples was performed by Dr Jose Esequiel Martin (Senior bioinformatician, Department of Medical Genetics, Cambridge University). Raw binary base call (BCL) files were demultiplexed and converted to fastq format using Illumina’s bcl2fastq 2.19, which also trimmed from the reads the indexes and the adaptors used for sequencing. All sample pairs (germline and tumour) were aligned to the hg38 version of the reference human genome using bwa 0.7.15 in alt contig aware mode as described by the authors (147). The generated SAM file was compressed into a BAM file and sorted by genomic position using samtools 1.3.1 (148). The sorted BAM files were subject to Base Quality Score Recalibration and Indel Realignment as specified in the Genome-Analysis Toolkit (GATK) (149) best practices (150)(151). For somatic variant calling the following GATK’s MuTect2 was used. A panel of normals (PON) was generated using the germline samples with GATK’s (version 4.0.3.0) *Mutect2* and *CreateSomaticPanelOfNormals* algorithms. Variants were called in all tumours using the PON and the matched germline sample with the GATK’s *MuTect2* algorithm to generate a VCF file (152). The percentage of contamination for each tumour sample was calculated using GATK’s *GetPileupSummaries* and *CalculateContamination* algorithms. Finally, the VCF files were filtered with GATK’s *FilterMutectCalls* algorithm. The resulting VCF file was annotated using annovar (153).
2.6 Germline genetic sequencing analysis of clinical samples

DNA was extracted from peripheral blood samples according to standard protocols. Next generation sequencing of a clinical gene panel including; SDHA, SDHB, SDHC, SDHD, KIT and PDGFRA (for GIST) and SDHA, SDHB, SDHC, SDHD, SDHAF2, MAX, TMEM127, VHL, RET, FH for (PPGL) was performed by the laboratory staff at Cambridge University Hospital NHS Foundation Trust or Birmingham Women’s and Children’s Hospital NHS Trust using the TrusightOne or Trusight Cancer sequencing panels (Illumina Inc., UK).

An average coverage depth of >20 fold was achieved for 98% of the regions sequenced. All detected variants were confirmed by Sanger sequencing. Whole exon deletions and duplications and large rearrangements are not detected using this method and multiple ligation probe analysis (MLPA) was performed for VHL, SDHB, SDHC and SDHD, in cases in whom a mutation in one of these genes was suspected but no mutation was detected by next generation sequencing.
Chapter 3

*In silico* protein prediction models and SDHB immunohistochemistry
Chapter 3.0: In silico protein prediction models and SDHB immunohistochemistry

3.11: Interpreting variants of uncertain significance in SDHx
3.12: In silico prediction models as a tool for confirming SDHx variant pathogenicity
3.13: A brief introduction to the integrated computational tool; DUET
3.14: Immunohistochemistry as a diagnostic and functional assessment tool
3.15: SDHB immunohistochemistry

3.2 Aims

3.3 Methods

3.31: Case Series
3.32: Literature Review
3.33: Variant assessment
3.34: Modelling of the SDHA/B/C/D complex and prediction of the effects of missense substitutions
3.45: SDHB Immunohistochemistry

3.4: Evaluating the structural effects of SDHA missense substitutions using a novel SDHA in silico protein prediction model

3.41: SDHA associated tumorigenesis
3.42: Identification of novel SDHA missense variants in UK cohort and Literature

3.43: In silico structural analysis of novel germline and somatic SDHA variants associated with tumorigenesis
3.44: Correlating the structural impact of 18 SDHA missense variants with the prediction of other computational tools and classification of 18 SDHA missense variants as per ACMG guidelines
3.45: *In silico* structural analysis of germline and somatic SDHA variants in control datasets

3.5: SDHB Immunohistochemistry and its role in the early detection of SDH deficient disease and classification of *SDHx* variant pathogenicity

3.5.1: Validating SDHB Immunohistochemistry for clinical diagnostic use

3.5.2: SDHB Immunohistochemistry in non PPGL SDH deficient disease

3.6: Discussion

3.7: Conclusion
Introduction

3.11 Interpreting variants of uncertain significance in SDHx

The terminology used in reference to genetic sequence abnormalities has recently been updated. In the past the word ‘mutation’ was used to describe an alteration in a nucleotide sequence that was believed to be pathogenic and the term ‘polymorphism’ was used to describe a common genetic alteration that was not felt to be disease causing. Recent recommendations from the American College of Medical Genetics and Genomics (ACMG), has called for the word ‘variant’ to be used to describe genetic sequence alterations with the following modifiers applied to further assign pathogenic risk; i) pathogenic, ii) likely pathogenic, iii) uncertain significance, iv) likely benign and v) benign (154). The criteria for assigning pathogenicity of variants have been ranked from ‘very strong’ to ‘supporting’ (Table 3.01) and the classification of a variant as ‘pathogenic’ or ‘likely pathogenic’ requires a combination of ‘very strong’ or ‘strong criteria’ in addition to ‘moderate’ or ‘supporting criteria’ (154).

As the most common type of SDHx variants associated with disease are missense variants (87), applying the ACMG classification to novel missense SDHx variants is challenging for a number of reasons. Firstly the penetrance of pathogenic SDHB variants is now considered to be considerably lower than previously believed (30%) (124), similarly a recent large Dutch study has demonstrated that the penetrance of SDHA variants s is estimated at <10% by the age of 70 years in asymptomatic non-proband carriers (155). Thus the interpretation of the contribution of a putative novel germline SDHx variant to the observed phenotype, is less likely to be aided by familial segregation studies.
Table 3.01: Table adapted from the ‘Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (154)’.

PVS = very strong evidence of pathogenicity, PS = strong evidence of pathogenicity, PM = moderate evidence of pathogenicity, PP = supporting evidence of pathogenicity

<table>
<thead>
<tr>
<th>Evidence of pathogenicity</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Very Strong</strong></td>
<td>PVS1: null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease</td>
</tr>
<tr>
<td><strong>Strong</strong></td>
<td>PS1: Same amino acid change as a previously established pathogenic variant regardless of nucleotide change</td>
</tr>
<tr>
<td></td>
<td>PS2: De novo (both maternity and paternity confirmed) in a patient with the disease and no family history</td>
</tr>
<tr>
<td></td>
<td>PS3: Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene</td>
</tr>
<tr>
<td></td>
<td>PS4: The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls</td>
</tr>
<tr>
<td><strong>Moderate</strong></td>
<td>PM1: Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation</td>
</tr>
<tr>
<td></td>
<td>PM2: Absent from controls (or at extremely low frequency if recessive) (in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium</td>
</tr>
<tr>
<td></td>
<td>PM3: For recessive disorders, detected in trans with a pathogenic variant</td>
</tr>
<tr>
<td></td>
<td>PM4: Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants</td>
</tr>
<tr>
<td></td>
<td>PM5: Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before</td>
</tr>
<tr>
<td></td>
<td>PM6: Assumed de novo, but without confirmation of paternity and maternity</td>
</tr>
<tr>
<td><strong>Supporting</strong></td>
<td>PP1: Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease</td>
</tr>
<tr>
<td></td>
<td>PP2: Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease</td>
</tr>
<tr>
<td></td>
<td>PP3: Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)</td>
</tr>
<tr>
<td></td>
<td>PP4: Patient’s phenotype or family history is highly specific for a disease with a single genetic etiology</td>
</tr>
<tr>
<td></td>
<td>PP5: Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation</td>
</tr>
</tbody>
</table>
Furthermore the presence of an $SDHx$ variant in control populations does not always exclude pathogenicity, as $SDHA$ variants in particular (e.g. c.91C>T p.Arg31*) can occur in healthy individuals at a population frequency of between 1/1000 and 1/10,000. Interestingly a high variant density has been identified for $SDHA$ in African American samples (156). This increased variant expression was initially attributed to higher rates of gene recombination, however a study using a high resolution recombination map have disputed this theory as a low recombination rate at the locus of the $SDHA$ gene was observed (157). It is now considered more likely that the four known $SDHA$ pseudogenes have contributed to increased $SDHA$ variant density by illegitimate recombination or gene conversion at the time of meiosis.

Focusing on functional testing, the $SDHx$ genes are tumour suppressor genes and therefore follow the Knudson ‘two-hit hypothesis’, requiring a mutation in both alleles for initiation of tumorigenesis. To date, data generated from mouse models have not supported the role of $SDHB$/$SDHD$ haploinsufficiency in tumorigenesis (158). Loss of heterozygosity (LOH) has been identified as one of the most common mechanisms accounting for the ‘second hit’ and is a useful and well documented functional method for confirming variant pathogenicity (159). However, LOH appears to be a less common mechanism causing loss of activity of the wild-type allele in SDH deficient disease and this has been demonstrated in particular for $SDHD$ (160) and $SDHA$ (98) related disease. A study evaluating the frequency of second allele inactivation in syndromic phaeochromocytoma, identified that LOH accounted for second allele inactivation in 80% of $SDHB$ related phaeochromocytoma tumours but only 50% of $SDHD$ related tumours (160). Similarly, in a study analysing $SDHA$ related WT GIST, the minority (36%) of tumours exhibited LOH (98). In those cases where inactivation of the wild type allele is not identified, further analysis is required to determine the effect of
methylation or inactivation of other genes on the activity of the wild type allele and tumour development. Therefore for SDHx variants, it would seem that alternative lines of functional analysis in addition to detailed phenotype and computational evidence is necessary in order to determine the pathogenicity of novel SDHx missense variants and in order to fulfill the ACMG classification criteria.

3.11: In silico prediction models as a tool for confirming SDHx variant pathogenicity

As sequencing technologies advance, the number of novel variants identified is growing exponentially and evaluation using functional experimental techniques is laborious and incapable of keeping pace. This challenge has prompted the development of several publically and commercially available computational tools designed to aid in the interpretation of novel variants. These computational tools use a combination of algorithms, which aim to predict the effect of a sequence variant at a nucleotide level and an amino acid level (161). Determining the effect of a novel missense variant on protein function is dependent on a number of criteria including i) whether the amino acid residue is highly conserved, ii) the biochemical impact of the nucleotide substitution and iii) the local impact within the protein structure and one or a combination of these consequences is considered in many of the available in silico prediction tools. Examples of these tools include; Polyphen (162), Mutalyser (163), Sorting Intolerant from Tolerant (SIFT)(164) and Mutpred (165), which are widely used to predict the effect of a missense variant on protein stability.

In general it is accepted that even the most reliable computational tools have a limited sensitivity estimated at 65-80%, when known disease variants were tested (166) and studies have also demonstrated that these tools lack specificity as well as sensitivity and that there is a risk that certain computational tools may over predict the damaging or deleterious consequences of missense variants (167) and therefore guidelines from the ACMG advocate
the combined use of multiple computational tools, in order to improve the accuracy of prediction (154). These computational tools have also been associated with reduced sensitivity in the interpretation of \textit{SDHx} variants and a number of studies across different \textit{SDHx} associated phenotypes report a discordance between functional assessment of variant pathogenicity and computational bioinformatic predictions (114)(168)(169). This reduced sensitivity has been attributed to the preservation of \textit{SDHx} genes throughout species(170)

3.12: A brief introduction to the integrated computational tool; DUET

In an effort to improve accuracy and dependability of predictive computational tools, an integrated computational approach called DUET was developed by Pires and colleagues(171) and is available as a publically available server (http://bleoberis.bioc.cam.ac.uk/duet). This integrative tool combines two methods; i) Site Directed Mutator (SDM) which compares the affinity of amino acid residues for the wild-type and mutated protein in the folded and unfolded state and then calculates the differential energy expenditure between the wild-type and mutant protein(172) and ii) Cutoff scanning matrix (mCSM), which applies machine learning, using structural signatures to predict the effects of missense variants (173). This combined computational tool has demonstrated superiority compared to other integrated computational methods (171). In more recent years DUET has also incorporated a further tool mCSM-Lig, which enables the prediction of the effect that a given missense variant will have on protein-small molecule interactions (174), further improving the sensitivity of this tool.
3.13: Immunohistochemistry as a diagnostic and functional assessment tool

Immunohistochemistry refers to the study of selective antigen expression in tissue by applying the corresponding binding antibody. Historically, immunohistochemistry (IH) was utilised for diagnostic purposes by pathologists but in more recent times, IH has emerged as a valuable molecular diagnostic tool with several potential applications including; i) tumour subtyping, ii) tumour prognosis, iii) identifying hereditary disease and iv) determining the benefit of specific targeted treatments (175).

Recent advances in the generation of monoclonal antibodies has also improved the sensitivity of IH techniques to detect single amino acid changes in a specific antigen and has facilitated the detection of the common oncogenic driver $BRAF$ V600E, which is now routinely screened for by IH in cancer including melanoma and thyroid carcinoma (176). Furthermore IH can guide hereditary gene testing such as IH testing for mismatch repair gene defects in gastric/endometrial or colorectal tumours in patients with suspected Lynch syndrome (177). In addition to detecting genetic alteration, IH also plays a valuable role in the functional assessment of variants of uncertain significance and therefore IH provides an excellent complementary tool to DNA sequencing and gene expression profiling.

3.14. SDH immunohistochemistry

Bi-allelic inactivation of any of the $SDHx$ genes, most commonly results in destabilisation of the SDH enzyme complex, which can be detected by loss of staining for the SDHB protein on IH (178). Negative immunostaining for SDHB is now a validated test for diagnosing likely pathogenic germline variants in $SDHA/SDHB/SDHC/SDHD$ and provides a reasonable assessment of pathogenicity of an identified novel $SDHx$ variant (7). In 2009, the first study using IH to detect pathogenic variants in $SDHB$, $SDHC$ and $SDHD$ was published (179).
SDHB immunohistochemistry was used as surrogate marker for any $SDHx$ variant as the SDHB protein is an anchor protein in the SDH complex, therefore a variant in any of the $SDHx$ genes is hypothesised to cause loss of SDHB immunoexpression. This study demonstrated excellent reliability for two different SDHB antibodies, across two independent institutions. Results of IH correlated with germline mutational status of the patient and with Western blot and SDH enzyme activity results (179). Importantly this study also determined that SDHB immunonegativity occurred in the presence of different $SDHB$ variants including missense, truncating and deleterious variants (179). Further studies have demonstrated the benefit of SDHB IH as a triage test for the detection of hereditary $SDHx$ related disease and also identified that the sensitivity of SDHB IH may be poorer in the detection of $SDHD$ mutated disease(7)(179). Tumours harbouring a variant in the $SDHA$ gene are also associated with a higher risk of false negative testing using SDHB IH as staining can show a diffusely weak pattern(178).

In 2011, application of an SDHA antibody test directed against the SDHA protein identified that loss of expression of SDHA on IH predicts pathogenic variants in the $SDHA$ gene and this was demonstrated for both truncating and missense variants (180). Therefore utilising both SDHB and SDHA IH should demonstrate loss of SDHB protein on IH but preservation of the SDHA protein in patients with pathogenic variants in $SDHB/SDHC/SDHD$ and loss of both SDHB and SDHA expression in $SDHA$ mutated tumours. A specific IH test for the $SDHD$ gene was reported in a retrospective study in 2015, and this study used a combination of SDHB and SDHD IH. Surprisingly, those tumours harbouring a variant in $SDHD$ were immunonegative for SDHB but demonstrated preservation of the SDHD protein compared to those tumours that did not harbour a variant in $SDHD$, where expression of SDHD was absent (181).
SDHB IH has been the most widely adapted IH test for SDH deficiency in clinical practice and serves a number of important clinical applications including; i) the early diagnosis of SDH deficiency in specific phenotypes such as PPGL, wild type GIST and specific histological subtypes of RCC, ii) SDHB IH provides additional prognostic information regarding malignant risk associated with PPGL (144), iii) it serves as a rapid and cost effective functional tool for the assessment of $SDHx$ variant pathogenicity and iv) SDHB IH can also identify those tumours that are SDH deficient due to a pathogenic somatic variant or those tumours harbouring an epimutation in the $SDHC$ gene (106). Despite the recognised clinical utility of this tool and advantages in terms of timing, one must remain aware of the limitations of this diagnostic tool. Limitations include inter-observer variation and the risk of false negative results particularly notable for $SDHA$ and $SDHD$ mutated tumours (178)(7) and finally the risk of false negatives with certain missense variants in the $SDHx$ genes, which may affect enzyme activity without affecting protein expression.
3.2: Aims:

In contrast to variants in the other subunits of SDH, relatively little information is available about \textit{SDHA} and its associated clinical phenotype. Since \textit{SDHA} variants were initially associated with PCC/PGL in 2011\(^{61}\), the spectrum of associated tumours expanded to also include GIST\(^{125}\), renal tumours\(^{182}\) and pituitary adenoma\(^{183}\). Additionally recent studies have demonstrated that \textit{SDHA} variants are associated with a risk of malignant disease\(^{125}(184)(185)\), thus the detection of a rare putative \textit{SDHA} variants may have clinical significance.

1) The aim of this study was to better characterise \textit{SDHA} variants detected in a UK cohort and in the literature, using an integrated computational approach DUET\(^{171}\) and to correlate the results of this computational testing with results from other in silico prediction models, in order to provide a comprehensive catalogue of \textit{SDHA} variants classified when possible according to the ACMG criteria\(^{154}\) for a pathogenic or benign variant.

2) To evaluate the role of SDHB IH as a functional assessment tool in SDH deficient disease
3.3 Methods

3.31: Case Series
Details of rare potentially pathogenic germline SDHA (OMIM: 600857, reference sequence: NG_012339.1) variants detected at UK NHS molecular diagnostic laboratories were obtained from those undertaking genetic testing. Referral data were collated on a standardized proforma and included: gender, age at presentation, method of presentation (sporadic vs. familial), location of tumour, presence of bilateral/multifocal disease, and evidence of malignancy.

3.32: Literature Review

A SDHA variant search in association with PCC/PGL, GIST, RCC, PA, Leigh syndrome, and optic atrophy was performed. This search was performed using the Human Gene Mutation Database (www.hgmd.cf.ac.uk), the Leiden Open Variation Database (http://www.lovd.nl/3.0/home), and publications indexed in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) up to May 2016. The following search terms were used: SDHA variant in combination with the terms: phaeochromocytoma, paraganglioma, GIST, pituitary adenoma, renal cell carcinoma, and the conditions Leigh syndrome and optic atrophy. Both germline and somatic variants were included in the search and the results were subcategorised for germline versus somatic variants identified.

3.33: Variant assessment

The criteria used to classify a variant included; review of the disease phenotype, the use of the predictive tools SIFT and Poly-Phen2 and when available, review of functional tumour studies (including immunohistochemical staining (IHC) of the SDHB/SDHA protein and loss
of heterozygosity studies (LOH)). The presence of the disease allele in a healthy control population was also confirmed by searching the EXAC database (http://exac.broadinstitute.org/). Variants identified in the literature which, were not considered to be disease causing by the authors were excluded. Information from computational predictive tools above and functional studies when available were combined with in silico predicted changes in protein stability and protein-protein affinity upon mutation for missense variants identified. This information was compiled and variants were classified as per the ACMG recommendations. An online genetic variation tool predictor (http://medschool.umaryland.edu/Genetic Variant_Interpretation_Tool1.html) based on ACMG guidelines was used to tabulate the evidence for the detected variants.

3.34: Modelling of the SDHA/B/C/D complex and prediction of the effects of missense substitutions

The modelling of the SDHA complex and the computational prediction of SDHA variants using DUET was performed by Dr David Ascher and I interpreted and correlated results with clinical phenotype and additional in silico and functional prediction analysis.

A molecular model of SDHA was generated using Modeller and Macro Model (Schrodinger, New York, NY) using the X-ray crystal structures of Succinate dehydrogenase flavoprotein subunit from the Avian respiratory complex ii (92% sequence identity; PDB ID: 1YQ4) (186) and the Flavoprotein subunit of Complex ii from Ascaris suum (72% sequence identity; PDB ID: 3VR8)(187). The effects of the variants upon the stability of SDHA were predicted using DUET(171).

To compare the in silico predictions for germline SDHA missense variants detected in patients with those not ascertained via diagnostic testing, I identified 24 rare (frequency
<0.01%) germline SDHA missense variants present in the ESP6500 cohort from Exome Variant Server (http://evs.gs.washington.edu) and correlated the effect of these missense variants on protein stability, complex formation, and ligand binding using our in silico prediction approaches.

In addition, eight presumed missense somatic SDHA variants detected in SDHx-related tumor types (seven renal cell carcinomas and one phaeochromocytoma) from the cBioPortal for cancer genomics (http://www.cbioportal.org), were evaluated.

3.35 SDHB Immunohistochemistry

Archival specimens of formalin-fixed paraffin-embedded (FFPE) tissues were requested and clinical details and germline mutational status were collected on each patient after informed consent. SDHB immunohistochemistry was performed on 4μm sections of FFPE tissue, after appropriate selection of tissue blocks by an experienced pathologist (Dr Alison Marker, Dr Anne Warren and Dr Olivier Giger). Commercially available SDHB polyclonal rabbit antibody was used at a dilution of 1:300. Heat induced epitope retrieval was carried out using Leica heat retrieval solution.

For the initial validation study, a total of 20 paraffin embedded (FFPE) tumour samples were selected. The tumour samples included PPGL from patients with pathogenic SDHx germline variants and patients without an identified SDHx variant by next generation sequencing. Two independent and experienced pathologists (Dr Alison Marker and Dr Anne Warren) were blinded to the germline mutational status of the patients and independently interpreted the SDHB immunohistochemistry for each tumour sample. Two independent interpretations of the SDHB immunostaining was obtained and correlated with the known germline mutational
status of the patient. A positive control of confirmed *SDHB* mutated adrenal phaeochromocytoma and a negative control including normal adrenal tissue and an SDH intact phaeochromocytoma was used for the initial validation study and subsequent studies. Positive staining was defined as granular cytoplasmic staining displaying the same intensity as internal positive control and negative staining was defined as absent cytoplasmic staining in the presence of an internal positive control and an equivocal staining patterns was defined as a weak diffuse cytoplasmic staining pattern.

Following on from the validation SDHB IH study, all additional samples were interpreted simultaneously by three independent physicians (Dr Ruth Casey, Dr Alison Marker and Dr Olivier Giger) and immunostaining was interpreted as positive/negative or equivocal as per the criteria above.
3.4: Evaluating the structural effects of SDHA missense substitutions using a novel SDHA in silico protein prediction model

3.41: Identification of novel SDHA missense variants in UK cohort and Literature

Fifteen previously unreported patients with ten different germline SDHA variants were identified in our novel UK cohort (Table 3.02). Two variants had been reported previously: the common nonsense variant (c.91C>T, p.Arg31*) was observed in five patients and a c.1753C>T (p.Arg585Trp) missense variant in one patient. A novel truncating variant in c.1468G>T (p.Glu490*) was identified in one patient with a metastatic GIST tumour. Four further novel candidate missense variants, one frameshift variant and one splice acceptor variant were detected in six kindreds (see Table 3.02) (a novel missense variant, c.923C>T (p.Thr308Met) in exon 8 of SDHA was detected in two apparently unrelated patients).

On review of the literature, 17 unique germline SDHA variants were identified in 47 individuals from 45 kindreds (Table 3.03). Three recurrent germline variants were identified: c.91C>T (p.Arg31*) nonsense variant in 22 kindreds (23 affected individuals) and two missense variants: c.1753C>T (p.Arg585Trp) (in two kindreds and two affected individuals) and c.1765C>T (p.Arg589Trp) (in four kindred’s and four affected individuals).
Table 3.02: Clinical phenotype of patients with variants in SDHA in novel UK cohort

<table>
<thead>
<tr>
<th>Variant</th>
<th>Sex</th>
<th>Age</th>
<th>Category</th>
<th>Single/multiple</th>
<th>Secretory</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.91C&gt;T (p.Arg31*)</td>
<td>M</td>
<td>56</td>
<td>HNPGL</td>
<td>Single</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>c.91C&gt;T (p.Arg31*)</td>
<td>M</td>
<td>33</td>
<td>Abdominal PGL</td>
<td>Single</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td>c.91C&gt;T (p.Arg31*)</td>
<td>M</td>
<td>45</td>
<td>Abdominal PGL</td>
<td>Single</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>c.91C&gt;T (p.Arg31*)</td>
<td>F</td>
<td>15</td>
<td>Adrenal PCC</td>
<td>Single</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>c.133G&gt;A (p.Ala45Thr)</td>
<td>M</td>
<td>36</td>
<td>Thoracic PGL</td>
<td>Single</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>c.136G&gt;A (p.Lys46Glu)</td>
<td>F</td>
<td>12</td>
<td>Abdominal PGL</td>
<td>Single</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>c.923C&gt;T (p.Thr308Met)</td>
<td>F</td>
<td>43</td>
<td>Thoracic PGL</td>
<td>Single</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>c.923C&gt;T (p.Thr308Met)</td>
<td>M</td>
<td>52</td>
<td>HNPGL</td>
<td>Multiple</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>c.1273G&gt;A (p.Val425Met)</td>
<td>M</td>
<td>62</td>
<td>PC and Paraspinal PGL</td>
<td>Multiple</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>c.1338delA (p.His447Metfs*23)</td>
<td>F</td>
<td>48</td>
<td>HNPGL</td>
<td>Single</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>c.1468G&gt;T (p.Glu490Ter)</td>
<td>M</td>
<td>32</td>
<td>GIST</td>
<td>Single</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1753C&gt;T (p.Arg585Trp)</td>
<td>F</td>
<td>34</td>
<td>PGL</td>
<td>Single</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>c.1765C&gt;T (p.Arg589Trp)</td>
<td>F</td>
<td>42</td>
<td>GIST</td>
<td>Single</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>c.1909-2A&gt;G</td>
<td>F</td>
<td>31</td>
<td>GIST</td>
<td>Single</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
### Table 3.03: SDHA variants (both germline and somatic identified in the literature)

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Protein change</th>
<th>Effect on protein</th>
<th>Germline</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.2T&gt;C</td>
<td>(p.Met1Thr)</td>
<td>Translation</td>
<td>Yes</td>
</tr>
<tr>
<td>c.91C&gt;T</td>
<td>(p.Arg31*)</td>
<td>Truncating</td>
<td>Yes</td>
</tr>
<tr>
<td>c.113A&gt;T</td>
<td>(p.Asp38Val)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.160C&gt;T+</td>
<td>(p.Gln54*)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.800 C&gt;T+</td>
<td>(p.Gln185*)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.457-2_c457delAGC</td>
<td>(p.Leu153Lysfs*71)</td>
<td>Truncating</td>
<td>Yes</td>
</tr>
<tr>
<td>c.457-3_c457-1_delCAG</td>
<td>N/A</td>
<td>Splice site</td>
<td>Yes</td>
</tr>
<tr>
<td>c.511C&gt;T</td>
<td>(p.Arg171Cys)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.553C&gt;T</td>
<td>(p.Gln185*)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.562C&gt;T</td>
<td>(p.Arg188Trp)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.622-2_622-2delA</td>
<td>N/A</td>
<td>Splice site</td>
<td>No</td>
</tr>
<tr>
<td>17k bp SDHA homozygous deletion on chr 5p.15</td>
<td>N/A</td>
<td>Truncating</td>
<td>No</td>
</tr>
<tr>
<td>c.725_736del+ c.989_990insTA</td>
<td>N/A</td>
<td>Truncating</td>
<td>No</td>
</tr>
<tr>
<td>c.767C&gt;T</td>
<td>(p.Thr256Ile)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.818C&gt;T</td>
<td>(p.Thr273Ile)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1046-1047delTG</td>
<td>(p.Leu349Arg fs*11)</td>
<td>Truncating</td>
<td>No</td>
</tr>
<tr>
<td>c.1151C&gt;G</td>
<td>(p.Leu349Arg fs*11)</td>
<td>Truncating</td>
<td>No</td>
</tr>
<tr>
<td>c.1255G&gt;A</td>
<td>(p.Gly419Arg)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.1334C&gt;T</td>
<td>(p.Glu491*)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.1361 C&gt;A</td>
<td>(p.Ala454Glu)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.1471G&gt;T</td>
<td>(p.Glu491*)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.1534C&gt;T</td>
<td>(p.Glu564Lys)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.1690G&gt;A</td>
<td>(p.Glu564Lys)</td>
<td>Missense</td>
<td>No</td>
</tr>
<tr>
<td>c.1753C&gt;T</td>
<td>(p.Arg589Trp)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1765C&gt;T</td>
<td>(p.Arg589Trp)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1766 G&gt;A</td>
<td>(p.Arg589Gln)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1794G&gt;C</td>
<td>(p.Lys598Asn)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1795-1G&gt;T</td>
<td>N/A splicing</td>
<td>Splice site</td>
<td>Yes</td>
</tr>
<tr>
<td>c.1873C&gt;T</td>
<td>(p.His625Tyr)</td>
<td>Missense</td>
<td>Yes</td>
</tr>
</tbody>
</table>
3.42: *In silico* structural analysis using DUET of novel germline and somatic SDHA variants associated with tumorigenesis

The most destabilizing germline variant predicted by DUET was the missense variant c.1766G>A (p.Arg589Gln), which had a score of 1.81 kcal/mol. This missense variant was detected in a single case of GIST in the literature (188). The second highest DUET score was associated with the germline missense variant c.1765C>T (p.Arg589Trp) which was identified in one patient in our study cohort with a GIST, and has been identified in the literature in one other patient with GIST (189) and two patients with paragangliomas (61).

Overall the most destabilizing variant predicted by DUET was a somatic variant (c.1361C>A p.Ala45Glu) identified in a single case of GIST in the literature, and had a score of 3.1 kcal/mol (169). Interestingly this variant was associated with loss of SDHB staining on immunohistochemistry but retained SDHA immunostaining. This *in silico* prediction tool, predicted that the variant was highly destabilizing. It is in the FAD binding pocket and the mutation would abolish FAD binding and disrupt formation of the succinate complex.

No significant differences were detected between DUET scores of missense variants associated with GIST and with PCC/PGL (P = 0.2). The *in silico* prediction tool predicted that 8/18 missense variants analyzed would have a mild or no effect on protein stability. Two of the eight variants were somatic variants; c.113A>T (p.Asp38Val) identified in a single GIST in the literature (169) and the c.1334C>T (p.Ser445Leu) variant, also detected in a single GIST in the literature (169). The remaining six variants were germline and four of the six variants were identified in our novel UK cohort; c.133G>A (p.Ala45Thr), c.136A>G (p.Lys46-Glu), c.923 C>T (p. Thr308Met), c. 1273G>A (p.Val425-Met). A potential alternative mechanism for pathogenicity could be postulated for three N-terminal missense substitutions: c.113A>T (p.Asp38Val), c.133G>A (p.Ala45Thr) and c.136A>G
(p.Lys46Glu), which were predicted to affect the transit peptide and potentially alter protein localization. One variant, c.1690G>A (p.Glu564Lys), was predicted to destabilize complex formation by mCSM-PPI (score of 0.951 kcal/mol).

3.43: Correlating the structural impact of 18 SDHA missense variants with the prediction of other computational tools and classification of 18 SDHA missense variants as per ACMG guidelines

Data from the in silico protein stability and affinity predictions was collated with data from computational predictive analyses and tumor studies when available in order to classify the 18 identified missense variants as per the ACMG guidelines (154). 13/18 (72.2%) missense variants met the criteria for a pathogenic (or likely pathogenic) variant (see Table 3.04). Five variants (included four novel variants identified in our UK cohort and one variant identified from the literature) did not meet the criteria for a pathogenic mutation, and the supporting evidence was supportive of a likely benign variant for four variants. One variant was classified as a variant of uncertain significance (VUS) because of insufficient evidence to classify the variant as either benign or pathogenic.
Table 3.04: Classification of potential pathogenicity of SDHA missense variants identified in literature and novel UK cohort as per ACMG guidelines(154)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Effect</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.113A&gt;T p.(D38V)</td>
<td>Likely benign (II)</td>
<td>PP5, PP4, BP1, BP4, BS1</td>
</tr>
<tr>
<td>c.133G&gt;A p.(A45T)</td>
<td>Likely benign (II)</td>
<td>PP4, BP1, BP4, PS3</td>
</tr>
<tr>
<td>c.136A&gt;G p.(K46E)</td>
<td>Likely benign (II)</td>
<td>PP4, BP1, BP4</td>
</tr>
<tr>
<td>c.511C&gt;T p.(R171C)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP4, BP1, PP3</td>
</tr>
<tr>
<td>c.562C&gt;T p.(R188W)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.767C&gt;T p.(T256I)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.800C&gt;T p.(T267M)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.923C&gt;T p.(T308M)</td>
<td>VUS - not enough evidence</td>
<td>BP1, PP3</td>
</tr>
<tr>
<td>c.1255G&gt;A p.(G419R)</td>
<td>Likely pathogenic (III)</td>
<td>PP4, PP3, PS3, BP1</td>
</tr>
<tr>
<td>c.1273G&gt;A p.(V425M)</td>
<td>Likely benign (II)</td>
<td>BP1, PP4, BP4</td>
</tr>
<tr>
<td>c.1334C&gt;T p.(S445L)</td>
<td>Likely pathogenic (III)</td>
<td>BP1, PP3, PS3, PP4</td>
</tr>
<tr>
<td>c.1690G&gt;A p.(E564K)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP4, PP3, BP1</td>
</tr>
<tr>
<td>c.1753C&gt;T p.(R585W)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.1765C&gt;T p.(R589W)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP5, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.1766G&gt;A p.(R589Q)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP4, PP5, PP3, BP1</td>
</tr>
<tr>
<td>c.1794G&gt;C p.(K598N)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, PP5, BP1</td>
</tr>
<tr>
<td>c.1873C&gt;T p.(H625Y)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, PP5, BP1</td>
</tr>
</tbody>
</table>
The variant c.1873C>T (p.His625Tyr), classified as likely pathogenic as per ACMG (154), was not shown to have any effect on protein stability in our in silico analysis (Table 3.05). This variant was identified in a patient with a PGL (proband) and her son who was diagnosed with a pituitary adenoma (183). No loss of the wild type allele was demonstrated in the pituitary adenoma but loss of SDHA and SDHB immunostaining was demonstrated in both tumor types. However taking a closer look at this mutation at a molecular level, reveals that His625 establishes an intricate network of polar interactions, including ionic interaction with Asp135 and Asp289, a donor–pi interaction with a Gln288 and a main-chain to main-chain hydrogen bond with Arg642 (Figure 3.1). These would most likely be disrupted by the mutation to Tyr, destabilizing the protein.

Three variants classified as likely benign and the variant classified as a VUS were identified in our novel UK cohort. One limiting factor to this classification was that tumor tissue was not available and so the variants could not be fully assessed. The first two variants c.133G>A (p.Ala45Thr) detected in a patient with a thoracic PGL and the c.136A>G (p.Lys46Glu), identified in a patient with an abdominal PGL, were consistently predicted as benign variants across different computational analysis tools and occurred at a frequency of up to 0.03% in healthy controls (see Table 3.05). These variants were predicted as having a potential effect on the transit peptide but the DUET, mCSM-PPI and mCSM-Lig scores could not be calculated and there was no effect on the cofactor.
### Table 3.05: Structural Impact of 17 SDHA Missense substitutions on *in silico* protein models and correlation with other predictive tools

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Phenotype</th>
<th>DUET score (kcal/mol)</th>
<th>mCSM-PPI score (kcal/mol)</th>
<th>Effect on protein</th>
<th>Effect on co-factor</th>
<th>SIFT or PolyPhen prediction</th>
<th>Frequency of allele per 1000 healthy population (EXAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.113A&gt;T</td>
<td>GIST</td>
<td>NA</td>
<td>NA</td>
<td>Transit peptide.</td>
<td>No</td>
<td>Benign</td>
<td>21.7</td>
</tr>
<tr>
<td>p.(D38V)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.133G&gt;A</td>
<td>Thoracic PGL</td>
<td>NA</td>
<td>NA</td>
<td>Near transit peptide.</td>
<td>No</td>
<td>Benign</td>
<td>0.34</td>
</tr>
<tr>
<td>p.(A45T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.136G&gt;A</td>
<td>Abdominal PGL</td>
<td>NA</td>
<td>NA</td>
<td>Near transit peptide.</td>
<td>No</td>
<td>Benign</td>
<td>0.24</td>
</tr>
<tr>
<td>p.(K46E)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.511C&gt;T</td>
<td>GIST</td>
<td>-1.183</td>
<td>-0.592</td>
<td>Destabilises protomer and complex.</td>
<td>Yes</td>
<td>Damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(R171C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.562C&gt;T</td>
<td>GIST</td>
<td>-0.901</td>
<td>-0.235</td>
<td>Destabilises protomer</td>
<td>Yes</td>
<td>N/A</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(R188W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.767C&gt;T</td>
<td>GIST</td>
<td>-0.397</td>
<td>-0.397</td>
<td>Destabilises protomer and complex.</td>
<td>Yes</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(T256I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.800C&gt;T</td>
<td>GIST</td>
<td>0.77</td>
<td>-0.287</td>
<td>Substrate binding pocket</td>
<td>Yes</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(T267M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.923C&gt;T</td>
<td>HNPGL, Thoracic PGL</td>
<td>-0.498</td>
<td>-0.16</td>
<td>Mildly destabilises protomer and part of substrate binding site.</td>
<td>No</td>
<td>Benign</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(T308M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1255G&gt;A</td>
<td>GIST</td>
<td>-1.268</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>No</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(G419R)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1273G&gt;A</td>
<td>PGL</td>
<td>0.083</td>
<td>0</td>
<td>No effect</td>
<td>No</td>
<td>Probably Damaging</td>
<td>0.02</td>
</tr>
<tr>
<td>p.(V425M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1334C&gt;T</td>
<td>GIST</td>
<td>0.971</td>
<td>0</td>
<td>Stabilises</td>
<td>No</td>
<td>Probably</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.(S445L)</td>
<td>protomer</td>
<td>damaging</td>
<td>described</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>----------</td>
<td>---------------</td>
<td>-----------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1690G&gt;A</td>
<td>GIST</td>
<td>0.263</td>
<td>-0.951</td>
<td>Destabilises complex</td>
<td>Yes</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(E564K)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1753C&gt;T</td>
<td>PGL,PC</td>
<td>-1.09</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>No</td>
<td>Damaging</td>
<td>.002</td>
</tr>
<tr>
<td>p.(R585W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1765C&gt;T</td>
<td>GIST,PGL</td>
<td>-1.383</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>No</td>
<td>Damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(R589W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1766 G&gt;A</td>
<td>GIST</td>
<td>-1.81</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>No</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(R589Q)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1794G&gt;C</td>
<td>GIST</td>
<td>0.301</td>
<td>0</td>
<td>No effect</td>
<td>No</td>
<td>N/A</td>
<td>.016</td>
</tr>
<tr>
<td>p.(K598N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1873C&gt;T</td>
<td>1 PA, 1</td>
<td>0.059</td>
<td>0</td>
<td>No effect</td>
<td>No</td>
<td>N/A</td>
<td>Not described</td>
</tr>
<tr>
<td>p.(H625Y)</td>
<td>HNPGL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The variant c.923C>T (p.Thr308Met), was detected in two patients who are not known to be related. This variant was associated with a malignant mediastinal PGL in one patient and multiple PGL and a PCC in a second patient. This variant was predicted to be benign by SIFT and PolyPhen (Table 3.05), but has not been identified in healthy controls and was also found to mildly destabilize the protomer and substrate binding site and therefore is likely to affect protein stability. Thr308 establishes, apart from hydrophobic interactions, hydrogen bonds that would be lost by the substitution to Methionine, which could also induce steric clashes (Figure 3.2). Its proximity to the ligand FAD could also imply a change in substrate binding as well. However due to insufficient evidence, the default classification of this variant was VUS.

The final variant c.1273G>A (p.Val425Met), was detected in a patient with a spinal PGL and PCC. This variant was predicted to be pathogenic by SIFT and PolyPhen and is only present in .002% of healthy controls. However this variant was not found to impact on protein stability by our in silico prediction analysis. As functional studies were not performed on this variant the overall criteria for a likely pathogenic variant were not met and the classification was a likely benign variant as per ACMG (154).
**Figure 3.1** (Image provided by Dr David Ascher). This figure illustrates the polar interactions that would be affected if Histidine was substituted to Tyrosine at amino acid position 625.

![Image of polar interactions](image1)

**Figure 3.2**: (Image provided by Dr David Ascher). This figure demonstrates the potential effect the substitution of Thr308 to Methionine, and the proximity of this amino acid residue to the ligand FAD.

![Image showing potential effect](image2)
3.44: In silico structural analysis of germline and somatic SDHA variants in control datasets

If SDHA pathogenic variants are usually associated with a low penetrance phenotype it might be postulated that rare pathogenic variants might also be detected in the general population. We therefore analyzed 24 rare (<0.05%) missense variants reported in a control data set (EVS6500, http://evs.gs.washington.edu) but not present in patient cohorts for predicted effect on protein stability, protein–protein and protein–ligand affinity and found that most 75% (18/24) were predicted to have a destabilizing effect and 41.6% (10/24) were predicted to affect complex formation. The in silico predictions of DUET correlated with SIFT and Polyphen prediction tools for 58.3% (14/24) of the variants (Table 3.06). Additionally, eight presumed missense somatic SDHA variants detected in SDH-related tumor types (seven renal cell carcinomas and one phaeochromocytoma) from the cBioPortal for cancer genomics (http://www.cbioportal.org) were evaluated and 75% (6/8) of the somatic missense variants identified in RCC and 1 PCC tumor described in the cBioportal database were predicted to destabilize the protein (7/8) were predicted to be deleterious by SIFT/ Polyphen) (Table 3.07).
Table 3.06: In silico structural analysis of SDHA variants identified from EVS

<table>
<thead>
<tr>
<th>Variant</th>
<th>DUET score</th>
<th>mCSM-PPI score</th>
<th>Effect on protein</th>
<th>SIFT Polyphen</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.276G&gt;C (p.Lys92Asn)</td>
<td>-0.766</td>
<td>-1.112</td>
<td>Destabilises protomer and complex and substrate binding site</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.287C&gt;T (p.Thr96Ile)</td>
<td>0.341</td>
<td>-0.649</td>
<td>Destabilises complex</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.607A&gt;G (p.Thr203Ala)</td>
<td>-1.191</td>
<td>-0.144</td>
<td>Destabilises protomer</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.704T&gt;C (p.Ile235Thr)</td>
<td>-2.713</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.155C&gt;T (p.Ser52Phe)</td>
<td>-0.881</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.830C&gt;T (p.Thr277Met)</td>
<td>0.19</td>
<td>0</td>
<td>No effect</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.861C&gt;G (p.Cys287Trp)</td>
<td>-1.703</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.986G&gt;A (p.Arg329Gln)</td>
<td>-0.466</td>
<td>0</td>
<td>Mildly destabilises protomer</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1042A&gt;T (p.Thr348Ser)</td>
<td>-1.219</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1055G&gt;A (p.Arg352Gln)</td>
<td>-0.228</td>
<td>0</td>
<td>No effect</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1090 G&gt;A (p.Val364Ile)</td>
<td>-0.975</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Benign</td>
</tr>
<tr>
<td>c.1122 G&gt;T (p.Glu374Asp)</td>
<td>0.052</td>
<td>-0.471</td>
<td>Destabilises complex</td>
<td>Benign</td>
</tr>
<tr>
<td>c.1171 G&gt;A (p.Ala391Thr)</td>
<td>-0.736</td>
<td>-0.007</td>
<td>Destabilises protomer</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1352 G&gt;A (p.Arg451His)</td>
<td>-1.939</td>
<td>-0.774</td>
<td>Destabilises protomer, complex and cofactor binding</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1430 C&gt;T (p.Pro477Leu)</td>
<td>-0.393</td>
<td>-0.725</td>
<td>Destabilises complex</td>
<td>Benign</td>
</tr>
<tr>
<td>c.1492 A&gt;C (p.Lys498Gln)</td>
<td>-0.189</td>
<td>-0.535</td>
<td>Destabilises complex</td>
<td>Benign</td>
</tr>
<tr>
<td>c.1532T&gt;C (p.Leu511Pro)</td>
<td>-2.113</td>
<td>-1.079</td>
<td>Destabilises protomer and complex</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1597 C&gt;G (p.Gln533Glu)</td>
<td>0.073</td>
<td>0</td>
<td>No effect</td>
<td>Benign</td>
</tr>
<tr>
<td>c.1751 C&gt;T (p.Ala584Val)</td>
<td>-0.285</td>
<td>0</td>
<td>No effect</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1772 C&gt;T (p.Ala591Val)</td>
<td>-0.55</td>
<td>0</td>
<td>Mildly destabilises protomer</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1786G&gt;T (p.Asn596Tyr)</td>
<td>-0.597</td>
<td>0</td>
<td>Mildly destabilises protomer</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1951G&gt;A (p.Glu651Lys)</td>
<td>0.492</td>
<td>0</td>
<td>No effect</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1973C&gt;T (p.Pro658Leu)</td>
<td>-0.234</td>
<td>0</td>
<td>No effect</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1979 C&gt;G (p.Ala660Gly)</td>
<td>-0.793</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Probably damaging</td>
</tr>
</tbody>
</table>
Table 3.07: *SDHA* variants identified as somatic variants in related tumour types in CBioportal

<table>
<thead>
<tr>
<th>Variant</th>
<th>Tumour type</th>
<th>Duet score</th>
<th>mCSM-PPI score</th>
<th>Effect on protein</th>
<th>SIFT and polyphen prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>* (p.Met388Ile)</td>
<td>RCC</td>
<td>-0.083</td>
<td>-1.001</td>
<td>Destabilises complex</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>c.1367C&gt;T (p.Ser456Leu)</td>
<td>RCC</td>
<td>0.66</td>
<td>0</td>
<td>Destabilises co factor binding and substrate binding site</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1396G&gt;A (p.Ala466Thr)</td>
<td>RCC</td>
<td>-1.671</td>
<td>0</td>
<td>Destabilises protomer, complex, co factor binding and substrate binding site</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.1360G&gt;T (p.Ala454Thr)</td>
<td>RCC</td>
<td>-2.212</td>
<td>-0.955</td>
<td>Destabilises protomer, complex, co factor binding and substrate binding site</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>c.2044C&gt;T (p.Pro643Ala)</td>
<td>RCC</td>
<td>-1.416</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>* (p.Ile383Phe)</td>
<td>RCC</td>
<td>-1.924</td>
<td>-1.182</td>
<td>Destabilises protomer and complex formation</td>
<td>Damaging</td>
</tr>
<tr>
<td>c.17G&gt;A (p.Gly6Asp)</td>
<td>RCC</td>
<td>--------</td>
<td>--------</td>
<td>Transit peptide</td>
<td>Benign</td>
</tr>
<tr>
<td>c.2074C&gt;T (p.Asp653 Ala)</td>
<td>PC</td>
<td>-0.69</td>
<td>0</td>
<td>Destabilises protomer</td>
<td>Probably damaging</td>
</tr>
</tbody>
</table>

*Nucleotide sequence not reported on CBioportal (www.cbioportal.org) or Ensembl (www.ensembl.org)*
3.5: SDHB Immunohistochemistry and its role in the early detection of SDH deficient disease and classification of SDHx variant pathogenicity

3.51: Validating SDHB IH for clinical diagnostic use

At the time of the initial validation study, SDHB immunohistochemistry was not available as a diagnostic test in Cambridge University NHS Foundation Trust Hospital. The only inter-observer variation noted in the validation study was for case number 6 (Table 3.08). This patient had a truncating variant in SDHA (c.91C>T p.Arg31*) and the immunostaining in this case was interpreted as having a mild cytoplasmic blush and the staining pattern was classified as equivocal. Aside from this case, consensus was reached by both pathologists in the remaining 19/20 (95%) cases and the immunostaining also correlated with the germline status of the patient in all 19 cases. Notably SDHB IH showed good specificity for other hereditary tumours including two patients with a clinical diagnosis of neurofibromatosis type 1 and a further patient with a pathogenic RET variant and sensitivity of this test was upheld for missense variants as well as truncating SDHx variants.

One limitation of this validation study was that no SDHD mutated tumour was included in the analysis. Due to the positive predictive value of SDHB immunohistochemistry (95%) observed in this validation study, SDHB immunohistochemistry was introduced in Cambridge University NHS Foundation Trust in January 2016 as a clinically available diagnostic test for all PPGL tumours, WT GIST tumours and RCC with a unique morphology, consistent with SDHB deficient RCC as per 2013 ISUP Vancouver Classification (109).
Table 3.08: Clinical characteristics, germline status and SDHB immunostaining interpretation of 20 patients in validation study

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age</th>
<th>Pathogenic germline variant</th>
<th>Tumour type</th>
<th>SDHB IH interpretation by observer 1</th>
<th>SDHB IH interpretation by observer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>No SDH mutation</td>
<td>Adrenal PC and GIST</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>Clinical diagnosis of NF1</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>SDHB c.137G&gt;A(p.Arg46Gln)</td>
<td>Metastatic abdominal PGL</td>
<td>Negative SDHB immunostaining</td>
<td>Negative SDHB immunostaining</td>
</tr>
<tr>
<td>4</td>
<td>79</td>
<td>SDHC c.43C&gt;T (p.Arg15*)</td>
<td>Abdominal PGL and GIST</td>
<td>Negative SDHB immunostaining</td>
<td>Negative SDHB immunostaining</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>SDHB C.136C&gt;T (p.Arg46X)</td>
<td>Abdominal PGL</td>
<td>Negative SDHB immunostaining</td>
<td>Negative SDHB immunostaining</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>SDHA c.91C&gt;T (p.Arg31*)</td>
<td>Abdominal PGL</td>
<td>Weak positivity</td>
<td>Equivocal</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>RET c.1900T&gt;A (p.Cys634Ser)</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>SDHB c.590C&gt;G (p. Pro197Arg)</td>
<td>Head and neck PGL, recurrent</td>
<td>Negative SDHB immunostaining</td>
<td>Negative SDHB immunostaining</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
<td>SDHB c.268C&gt;T (p.Arg90*)</td>
<td>Abdominal PGL</td>
<td>Negative SDHB immunostaining</td>
<td>Negative SDHB immunostaining</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>11</td>
<td>68</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>12</td>
<td>64</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>13</td>
<td>64</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>14</td>
<td>51</td>
<td>SDHB c.268C&gt;T (p.Arg90*)</td>
<td>Metastatic abdominal PGL</td>
<td>Negative SDHB immunostaining</td>
<td>Negative SDHB immunostaining</td>
</tr>
<tr>
<td>15</td>
<td>45</td>
<td>No SDH mutation</td>
<td>Adrenal PC+GIST</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>16</td>
<td>28</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>17</td>
<td>63</td>
<td>No SDH mutation</td>
<td>Abdominal PGL</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>18</td>
<td>43</td>
<td>Clinical diagnosis of NF1</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>19</td>
<td>47</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
<tr>
<td>20</td>
<td>29</td>
<td>No SDH mutation</td>
<td>Adrenal PC</td>
<td>Positive SDHB immunostaining</td>
<td>Positive SDHB immunostaining</td>
</tr>
</tbody>
</table>
3.52: SDHB IH in non PPGL SDH deficient disease

In addition to the initial validation study, SDHB IH was carried out on a further 90 tumour samples, including 42 (46%) non PPGL tumour samples (Table 4.8). This study has included a combination of retrospective and prospective cases. The majority of the non PPGL tumour samples analysed have included WT GIST samples (31, 79%) and RCC (6, 14%) samples, however tumours including breast, thyroid, pituitary and 2 pancreatic neuroendocrine tumours, have also been analysed.

On review of the WT GIST samples, SDHB IH correlated with germline mutational status in 27/31 samples (87%) (Figure 3.3). One WT GIST tumour (case # 13) with a truncating SDHD variant, had a weak diffuse staining pattern and therefore the result was classified as equivocal. This pattern has previously been reported for SDHD variants using SDHB IH23. Insufficient tumour material was available to perform additional functional analysis but family segregation analysis identified a daughter with a carotid body tumour, suggesting the pathogenic role of the SDHD variant in this case. Loss of SDHB IH was identified in three cases of WT GIST (case #5, #6, #14) with no identifiable germline SDHx variant. All three cases were female and presented at a young age (mean age at presentation 18.8 years range (15-22)) and all patients developed metastatic disease. All three patients were subsequently demonstrated to have hypermethylation of the promoter region of the SDHC gene resulting in the so called ‘SDHC epimutation’. This somatic epimutation has been demonstrated as a cause for SDH deficient GIST (98) and previous studies have demonstrated that SDHB IH is an effective triage test for identifying SDH deficiency caused by this epimutation (7). This epimutation will be discussed further in chapter 8.

SDHB IH was performed on 6 RCC tumours and one SDH deficient RCC tumour was identified (case # 37, Table 3.09) and again the SDHB IH correlated with the germline
mutational status of the patient (SDHB c.79C>T p.Arg27*). SDHB IH was also performed on 5 further tumour samples (case #38-42, Table 3.09) and no loss of expression was identified in any of these tumour samples. Three of these patients had known pathogenic germline variants in the SDHB gene; patients (case # 38, 40, 41). This demonstrates the value of SDHB IH in determining the causative role of germline SDHx variants in less typical tumour phenotypes.

Looking at these cases in more detail;

1) Case #38, is a 56 year old lady with a previous history of a phaeochromocytoma and a known germline pathogenic variant in SDHB (c.540+1G>A). She had a thyroidectomy as treatment for thyrotoxicosis and histology revealed an incidental finding of a 1cm papillary thyroid carcinoma (PT1a Nx). SDHB immunostaining was performed to determine the role of the known germline SDHB mutation in the pathogenesis of the papillary thyroid cancer and SDHB IH showed preservation of the SDHB protein,

2) Case #40 is a 48 year old lady, who was diagnosed with a 6cm triple negative breast cancer and as part of her radiological staging for metastatic disease, an 8.5cm left phaeochromocytoma was diagnosed. Germline testing revealed a pathogenic variant in SDHB (c.380 T>G p.Ille127Ser). SDHB immunostaining was again performed to investigate the causative role of the SDHB mutation in the breast cancer and the immunostaining was strongly preserved.

3) Case # 41, is the case of an 80 year old male patient who presented with apoplexy secondary to a large non-functioning pituitary adenoma. Initial de-bulking was carried out because of optic chiasm compression. Five years later, this gentleman was diagnosed with a germline pathogenic variant in SDHB (c.380T>G p.Ille127Ser), after he underwent family screening following the diagnosis of a phaeochromocytoma and
a germline SDHB mutation in his nephew. Immunostaining of the pituitary tissue was positive for SDHB, indicating no loss of the SDHB protein in the pituitary tumour (Figure 3.5B).
Table 3.09: SDHB IH in non PPGL SDH deficient disease

<table>
<thead>
<tr>
<th>Case #</th>
<th>Phenotype</th>
<th>Germline mutation</th>
<th>SDHB IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>2</td>
<td>GIST</td>
<td>SDHB c.380G&gt;T p.(Ile127Ser)</td>
<td>Loss</td>
</tr>
<tr>
<td>3</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>4</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>5</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Loss</td>
</tr>
<tr>
<td>6</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Loss</td>
</tr>
<tr>
<td>7</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>8</td>
<td>GIST</td>
<td>SDHA c.1765C&gt;T p.(Arg589Trp)</td>
<td>Loss</td>
</tr>
<tr>
<td>9</td>
<td>GIST</td>
<td>SDHC c.380A&gt;G, p.(His127Arg)</td>
<td>Loss</td>
</tr>
<tr>
<td>10</td>
<td>GIST</td>
<td>SDHA c.1468G&gt;T p.(Glu490Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>11</td>
<td>GIST</td>
<td>SDHD c.34G&gt;A (VUS)</td>
<td>Loss</td>
</tr>
<tr>
<td>12</td>
<td>GIST</td>
<td>SDHD c.296delT, p.(Leu99fs)</td>
<td>Equivocal</td>
</tr>
<tr>
<td>13</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Loss</td>
</tr>
<tr>
<td>14</td>
<td>GIST</td>
<td>SDHC c.43C&gt;T p.(Arg15*)</td>
<td>Loss</td>
</tr>
<tr>
<td>15</td>
<td>GIST</td>
<td>SDHC c.148C&gt;T p.(Arg50Cys)</td>
<td>Loss</td>
</tr>
<tr>
<td>16</td>
<td>GIST</td>
<td>NF1 c.4421delG, p.(Tyr794Ter)</td>
<td>Preserved</td>
</tr>
<tr>
<td>17</td>
<td>GIST</td>
<td>NF1 c.5770delT, p.(Cys1924ValfsTer5)</td>
<td>Preserved</td>
</tr>
<tr>
<td>18</td>
<td>GIST</td>
<td>NF1 c.7706dupA, p.(His2569GlnfsTer6)</td>
<td>Preserved</td>
</tr>
<tr>
<td>19</td>
<td>GIST</td>
<td>NF1 c.4558delA, p.(Thr1520HisfsTer33)</td>
<td>Preserved</td>
</tr>
<tr>
<td>20</td>
<td>GIST</td>
<td>No mutation detected</td>
<td>Preserved</td>
</tr>
<tr>
<td>21</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>22</td>
<td>GIST</td>
<td>NF1 (Clinical diagnosis)</td>
<td>Preserved</td>
</tr>
<tr>
<td>23</td>
<td>GIST</td>
<td>NF1 (Clinical diagnosis)</td>
<td>Preserved</td>
</tr>
<tr>
<td>24</td>
<td>GIST</td>
<td>NF1 (Clinical diagnosis)</td>
<td>Preserved</td>
</tr>
<tr>
<td>25</td>
<td>GIST</td>
<td>NF1 (Clinical diagnosis)</td>
<td>Preserved</td>
</tr>
<tr>
<td>26</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>27</td>
<td>GIST</td>
<td>NF1 (Clinical diagnosis)</td>
<td>Preserved</td>
</tr>
<tr>
<td>28</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>29</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>30</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>31</td>
<td>GIST</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>32</td>
<td>RCC</td>
<td>Not tested</td>
<td>Preserved</td>
</tr>
<tr>
<td>33</td>
<td>RCC</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>34</td>
<td>RCC</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>35</td>
<td>RCC</td>
<td>TMEM127 mutation c.117_120delGTCT</td>
<td>Preserved</td>
</tr>
<tr>
<td>36</td>
<td>RCC</td>
<td>MAX mutation c.97C&gt;T (p. Arg33*)</td>
<td>Preserved</td>
</tr>
<tr>
<td>37</td>
<td>RCC</td>
<td>SDHB c.79G&gt;T p.Arg27*</td>
<td>Loss</td>
</tr>
<tr>
<td>38</td>
<td>Papillary thyroid cancer</td>
<td>SDHB c.540+1G&gt;A</td>
<td>Preserved</td>
</tr>
<tr>
<td>39</td>
<td>Pancreatic NET</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
<tr>
<td>40</td>
<td>Breast Ca</td>
<td>SDHB c.380T&gt;G p.Ile127Ser</td>
<td>Preserved</td>
</tr>
<tr>
<td>41</td>
<td>Pituitary adenoma</td>
<td>SDHB c.380T&gt;G p.Ile127Ser</td>
<td>Preserved</td>
</tr>
<tr>
<td>42</td>
<td>Pancreatic NET</td>
<td>No germline mutation</td>
<td>Preserved</td>
</tr>
</tbody>
</table>
**Figure 3.3:** Figure A) shows the spindle shaped morphology of a WT GIST tumour from case #20 with a pathogenic NF1 variant. Figure B) shows the same tumour after SDHB IH, showing preservation of the SDHB protein. Figure C) demonstrates a mixed epithelioid morphology in the WT GIST tumour of case #2 and figure D) shows loss of SDHB expression on IH of the same tumour, correlating with the germline Pathogenic variant in SDHB gene (c.380G>T p.(Ile127Ser) in case #2, figures courtesy of Dr Olivier Giger
**Figure 3.4:** This demonstrates the mixed epithelioid histology of the WT GIST tumour from case #5 in figure A) and case #6 in figure C). Both tumours show loss of SDHB IH as demonstrated in figure B) for case #5 and figure D) for case #6 and both cases were identified with a somatic $SDHC$ epimutation. Figures courtesy of Dr Olivier Goger.

![Figure 3.4](image)

**Figure 3.5:** A) demonstrates SDHB immunohistochemistry performed on RCC tumour tissue from Case #37. This figure shows normal kidney tissue in the upper half of the image with preservation of SDHB immunostaining (blue arrow) and loss of SDHB immunostaining in the RCC tumour tissue in the bottom half of the image (red arrow), image courtesy of Dr Anne Warren. Figure B) shows preservation of the SDHB protein on tumour tissue from a pituitary adenoma diagnosed in case #41, image courtesy of Dr Kieren Allinson.

![Figure 3.5](image)
3.53: SDHB IH as a tool for determining SDHx variant pathogenicity

As part of this IH analysis, we have also included 15/90 (16.6%) tumours that were i) referred for validation of variants of uncertain significance and ii) we also included when available, those tumours that had identified germline variants in SDHA in order to provide additional functional assessment of these gene variants.

Reviewing the SDHA mutated tumours, 12 tumour samples including 4 PPGL samples, one rib metastasis from a patient with a metastatic SDHA mutated paraganglioma and 7 WT GIST tumours, were analysed. The majority of these tumours (9/16, 60%) harboured the common truncating variant in SDHA c.91C>T (p.Arg31Ter), and all tumours aside from one PGL included in the validation study (case #6), harbouring this truncating variant had loss of SDHB expression on IH. Loss of SDHB IH was also demonstrated in two further tumours, one harbouring a missense variant SDHA (c.1468G>T p.Glu 490Ter) and another tumour from a patient with a germline splice variant in SDHA (SDHA c.1909-2A>G). However one WT GIST tumour had an equivocal result on SDHB IH, with a weak diffuse staining pattern and this tumour was from a patient with a germline missense variant in SDHA (c.1765C>T p.Arg589Trp). Interpretation of the SDHB IH, similar to other cases, was carried out by three independent physicians (AM, OG, RC), all blinded at the time to the mutational status of the patient and in this case agreement was reached on a result of equivocal staining by all three physicians. This variant was predicted to be damaging using DUET, with a score of -1.383 and was classified as likely pathogenic based on ACMG criteria (Table 3.04) and according to previous functional analysis performed by other groups(61)(189).
A phaeochromocytoma tumour from a 38 year old man (case #45, Table 3.10) was included in this analysis, as he was diagnosed with a germline variant in SDHD (c.14G>A P.Trp5Ter) and on family screening the variant was confirmed as being maternally inherited. As disease penetrance associated with SDHD variants is almost exclusively dependant on paternal transmission, SDHB immunostaining was performed to confirm the role of the maternally inherited SDHD variant in the pathogenesis of the phaeochromocytoma diagnosed in this case. Immunohistochemistry revealed absent SDHB immunostaining in the tumour (See Figure 3.6A). Further tumour sequencing in this case demonstrated LOH at the SDHD germline locus (Figure 3.6B), correlating with the SDHB IH result and again suggesting that the maternally inherited SDHD variant was causative in the development of PPGL in this case.

Finally we have also analysed two PPGL tumour samples from two unrelated individuals who were identified with a germline SDHB gene duplication. SDHB IH was performed on the two tumour samples to determine pathogenicity of this germline variant and preservation of the SDHB protein was identified in both tumours (Table 3.10)
**Figure 3.6:** SDHB IH in figure A) shows loss of SDHB expression in the phaeochromocytoma tumour from case #45 with a maternally inherited SDHD variant. Figure B) shows an image taken from Integrated Genomic Viewer (IGV) and shows a homozygous SDHD (c.14G>A p.Trp5Ter) variant in the tumour

**Table 3.10:** Variants assessed for pathogenicity using SDHB IH

<table>
<thead>
<tr>
<th>Case #</th>
<th>Phenotype</th>
<th>Pathogenic germline variant</th>
<th>SDHB IH</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>Phaeochromocytoma</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>44</td>
<td>Phaeochromocytoma</td>
<td>SDHB duplication</td>
<td>Preserved</td>
</tr>
<tr>
<td>45</td>
<td>Phaeochromocytoma</td>
<td>SDHB duplication</td>
<td>Preserved</td>
</tr>
<tr>
<td>45</td>
<td>Phaeochromocytoma</td>
<td>SDHD c.14G&gt;A p.Trp5Ter (maternally inherited)</td>
<td>Loss</td>
</tr>
<tr>
<td>47</td>
<td>Phaeochromocytoma</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>48</td>
<td>Paragangioma</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>49</td>
<td>Rib metastases</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>6*</td>
<td>Phaeochromocytoma</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Equivocal</td>
</tr>
<tr>
<td>50</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>51</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>52</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>53</td>
<td>GIST</td>
<td>SDHA c.91C&gt;T p.(Arg31Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>54</td>
<td>GIST</td>
<td>SDHA c.1765C&gt;T p.(Arg589Trp)</td>
<td>Equivocal</td>
</tr>
<tr>
<td>55</td>
<td>GIST</td>
<td>SDHA c.1468G&gt;T p.(Glu 490Ter)</td>
<td>Loss</td>
</tr>
<tr>
<td>56</td>
<td>GIST</td>
<td>SDHA c.1909-2A&gt;G</td>
<td>Loss</td>
</tr>
</tbody>
</table>

*= case also included in initial SDHB IH validation study
4.6: Discussion

The *in silico* prediction of *SDHA* variants using DUET in this study should aid interpretation of genetic testing results in patients with relevant tumour types identified with an *SDHA* variant. Most putative *SDHA* variants detected in patients presenting with a relevant tumour were demonstrated to impair protein stability and facilitated further classification of the variants according to ACMG criteria (154). I also found that many rare *SDHA* missense variants present in the ESP6500 exome sequencing data set were also predicted to be destabilizing by DUET and pathogenic by PolyPhen/SIFT. Though no information is available on the phenotype of ESP6500 individuals with *SDHA* variants, this comparison does illustrate the challenge in interpreting the significance of rare genetic variants in candidate genes. For affected individuals with putative *SDHA* missense mutations, this analysis would suggest that, in addition to in silico protein structure and bioinformatic predictions of pathogenicity (e.g. SIFT/ PolyPhen), additional functional studies should be undertaken to aid variant classification.

The low inter-observer variation in the interpretation of SDHB immunohistochemistry in our validation study, as well as the acknowledged benefit of this diagnostic tool in the detection of SDH deficiency due to germline *SDHx* mutation (7) or *SDHC* hyper methylation(7), has prompted the introduction of SDHB immunohistochemistry as a clinically available diagnostic test in Cambridge University Hospital NHS Foundation Trust. The SDHB immunostaining is reported as ‘preserved’ or ‘absent’ and if SDHB immunostaining is ‘absent’, an additional sentence is added to the report, which states that this finding is suggestive of SDH deficiency and recommends a clinical genetics referral.

In addition to its triaging role for SDH deficiency, SDHB immunostaining also has the potential to provide useful functional analysis in the interpretation of variants of uncertain
significance and in evaluating the causative role of SDH deficiency in atypical tumour phenotypes. In this study SDHB immunostaining has suggested that germline SDHx variants did not play a causative role in a i) non-functioning pituitary adenoma (case #41), ii) a papillary thyroid cancer (case#38) and iii) a breast cancer (case#40). It is a useful tool in the interpretation of variants of uncertain significance and has further aided our interpretation of SDHA variants in addition to other novel putative variants and in the interpretation of the causative role of a maternally inherited SDHD variant in the development of a phaeochromocytoma in case #45.

However, despite the demonstrated clinical and diagnostic utility of this test, some issues remain with regard to test sensitivity. This was notable in this study for certain SDHA and SDHD variants, as has previously been demonstrated in other studies (178)(7). It is also important to remember that IH studies can demonstrate heterogeneous expression patterns and this has been demonstrated for mismatch repair deficiency IH (190) and IH for PTEN mutations in Cowden syndrome (191). Therefore SDHB IH may not be always be an ‘all or nothing’ diagnostic tool. Acknowledging the potential pitfalls associated with SDHB IH, this study acts as a reminder of the importance of offering comprehensive genetic sequencing to patients with suspicious phenotypes regardless of the SDHB IH results.
3.7 Conclusion:

The combined *in silico* prediction and SDHB IH analyses has demonstrated that these tools have clinical utility in verifying variants of uncertain significance in the *SDHx* genes. However, both of these protein based assessment tools lack 100% sensitivity and this study has identified specific discrepancies in each tool and demonstrates the need to exercise caution when interpreting pathogenicity of novel rare sequence *SDHx* variants and that, in such cases, whenever possible a variety of strategies, including structural prediction analysis, molecular genetics, SDHB IH, and when possible functional tests exploring enzyme function or ‘oncometabolite’ accumulation such as; metabolomics analysis or methylome profiling of tumours should be performed, to better define the likelihood of pathogenicity of *SDHx* variants and to ensure optimum clinical management.
Chapter 4

Investigating the clinical and molecular features of Renal and Phaeochromocytoma/Paraganglioma Tumour Association Syndrome (RAPTAS)

DECLARATION: The findings reported in this study have been published in:

Chapter 4: Investigating the clinical and molecular features of Renal and Phaeochromocytoma/Paraganglioma Tumour Association Syndrome (RAPTAS)

4.1 Introduction

3.11 Hereditary renal cell carcinoma syndromes (RCC)
3.12 The role of SDHx in RCC
3.13 The co-occurrence of RCC and phaeochromocytoma

4.2 Aims

4.3 Methods

4.31 Case series
4.32 Molecular genetic testing of case series
4.33 Histology review
4.34 Tumour sequencing
4.35 Bioinformatics
4.36 Multiple primary tumour score
4.37 Literature review
4.38 Statistical analysis

4.4 Results

4.41 Literature review
4.42 Case series demographics and clinical features
4.43 Molecular Genetics Analysis of the non-VHL RAPTAS case series
4.44 Histology review
4.45 Tumour sequencing

4.5 Discussion

4.51 Role of clinical features in suggesting specific genes
4.52: Role of histology in suggesting specific genes in RAPTAS
4.53 Investigation of potential RAPTAS patients

4.6 Conclusion
4.1 Introduction

Renal cell carcinoma (RCC) is one of the ten most common forms of adult cancer worldwide and approximately 3% of RCC cases occur due to a hereditary disorder (192). RCC compromise a number of distinct histological subtypes and these are associated with different molecular genetic drivers. The most common histological subtype of RCC is clear cell (or conventional) RCC (ccRCC), accounting for 75% of all cases. Other histological sub-types include; papillary (10-15%), chromophobe (3-5%) and the remaining rare histological variants account for approximately 1-3% of all cases (193). Renal cell carcinoma is a recognised clinical feature of a number of hereditary cancer syndromes including; von Hippel-Lindau (VHL) disease, SDH deficient RCC, hereditary papillary renal carcinoma (HPRC), Birt-Hogg-Dube (BHD), hereditary leiomyomatosis renal cell carcinoma (HLRCC), BAP1 tumour predisposition syndrome, Cowden syndrome and, rarely, tuberous sclerosis (TSC) (194) (Table 3.1). Whilst VHL disease associated RCC is always clear cell type and MET activating mutations cause hereditary Type 1 papillary RCC in some inherited disorders a variety of histological subtypes may occur (e.g. BHD syndrome).

Recent guidelines recommend that specific criteria should be considered when evaluating the possibility of a hereditary RCC syndrome and these criteria include; i) age at presentation, ii) other clinical features of a hereditary cancer syndrome (e.g. retinal haemangioblastomas in VHL), iii) family history of RCC or features of relevant syndrome, iv) bilateral or multicentric RCC and v) rarer subtypes of non-clear cell RCC (194). A better understanding of the molecular drivers of RCC, in particular the role of the VHL gene in ccRCC, has revolutionised the therapeutic landscape in RCC over the past decade and has contributed to the large number of targeted therapies that are currently available for the treatment of advanced RCC (195).
Table 4.1: Recognised hereditary RCC syndromes, adapted from ‘Prevalence and Characteristics of Patients with Suspected Inherited Renal Cell Cancer: Application of the ACMG/NSGC Genetic Referral Guidelines to Patient Cohorts.’ (194)

<table>
<thead>
<tr>
<th>Hereditary syndrome</th>
<th>Histological subtype of RCC</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Hippel-Lindau</td>
<td>Clear cell RCC</td>
<td>VHL</td>
</tr>
<tr>
<td>Hereditary papillary renal carcinoma</td>
<td>Type 1 papillary RCC</td>
<td>MET</td>
</tr>
<tr>
<td>Hereditary leiomyomatosis and renal cell carcinoma</td>
<td>Type 2 papillary RCC/tubolopapillary</td>
<td>FH</td>
</tr>
<tr>
<td>Birt Hogg- Dubé</td>
<td>Chromophobe RCC; ccRCC</td>
<td>FLCN</td>
</tr>
<tr>
<td>Tuberous Sclerosis</td>
<td>Chromophobe RCC/renal oncocyctoma</td>
<td>TSC1,TSC2</td>
</tr>
<tr>
<td>Succinate dehydrogenase B deficiency-associated RCC</td>
<td>Eosinophilic RCC</td>
<td>SDHB</td>
</tr>
<tr>
<td>Cowden Syndrome</td>
<td>No specific histological subtype</td>
<td>PTEN</td>
</tr>
</tbody>
</table>

4.12 The role of SDHx in RCC

The first description of a non-PPGL neoplasia associated with pathogenic germline SDHx variants was made by Vanharanta and colleagues in 2004, and they described two kindreds with a known germline pathogenic variant in SDHB who were affected by early onset RCC (3). SDHB LOH was identified in all cases of RCC in this study and thus implicated SDHB variants in the development of early-onset hereditary kidney cancer for the first time.

Interestingly in this study, those patients identified with SDHB mutated RCC also had either a
co-existing diagnosis of a PPGL or a family history of PPGL (3). In 2008, Ricketts and colleagues investigated a cohort of 68 patients with a family history of RCC or early onset or multicentric RCC, for a suspected hereditary predisposition and identified three patients with a pathogenic germline \textit{SDHB} variant (108). Therefore this study identified a role for \textit{SDHB} germline variants in hereditary RCC even in the absence of a personal or family history of PPGL (108).

It is now estimated that SDH deficient RCC account for 0.5% of all RCC cases (103) and the \textit{SDHB} gene is the most common of the \textit{SDH}x subunit genes to be implicated in hereditary RCC (though all of the \textit{SDH}x subunit genes have now been implicated in hereditary RCC) (105) (106) (107).

4.13: The co-occurrence of RCC and phaeochromocytoma

Causes for the occurrence of different tumour types in the same individual or in close relatives may include shared environmental exposures and/or inherited neoplasia disorders. Combinations of specific tumour types may strongly implicate specific inherited cancer syndromes (196). Thus the combination of phaeochromocytoma (PC) and renal cell carcinoma (RCC) was recognised as a ‘form fruste’ of von Hippel-Lindau (VHL) disease more than 60 years ago (197). The proportion of PPGL cases attributable to a genetic cause is at least 10-fold higher than for RCC. Germline variants in at least 19 different genes have been reported to predispose to one or more of these tumour types: PC/PGL/HNPGL only (\textit{NF1}, \textit{RET}, \textit{MAX}) (198), RCC only (\textit{BAP1}, \textit{CDC73}, \textit{CDKN2B}, \textit{FLCN}, \textit{MET}, \textit{PBRM1}, \textit{PTEN}, \textit{TSC1}, \textit{TSC2}) (194) (195) or both PC/PGL/HNPGL and RCC (\textit{FH}, \textit{SDHA}, \textit{SDHB}, \textit{SDHC}, \textit{SDHD}, \textit{TMEM127}, and \textit{VHL}) (105) (106) (107) (66) (199). However, with the exception of VHL disease and, to a lesser extent, \textit{SDHB} variants other reported genetic causes are rare and clinical and molecular studies are limited mostly to anecdotal case reports.
4.2: Aims

1. To investigate the genetic architecture of the clinical association (in the same individual or family) of a renal tumour and PPGL without evidence of VHL disease (herein referred to as non-VHL RAPTAS (Renal and Phaeochromocytoma/Paraganglioma Tumour Association Syndrome).

2. To better inform genotype phenotype correlations associated with non VHL RAPTAS

3. Provide guidelines for genetic testing in patients with suspected non VHL RAPTAS

4.3 Methods

4.31 Case series

Details of patients referred for molecular genetic testing because of a suspected hereditary cause of PC/PGL or RCC over a period of 15 years (2001-2016) were reviewed and those with clinical (e.g. in addition to PC/RCC the presence of retinal or CNS hemangioblastoma, multiple renal or pancreatic cysts, pancreatic neuroendocrine tumours or endolymphatic sac tumours) or molecular evidence of VHL disease were excluded. Patients included had either (i) a personal history of PC/PGL/HNPGL and a renal tumour or ii) or PC/PGL/HNPGL and RCC were present in first degree relatives (e.g. PC in a proband and RCC in a parent).

Patients meeting these criteria were classified as having “non-VHL RAPTAS”. Referral data from three UK NHS molecular diagnostic laboratories undertaking genetic testing, were collated on a standardised proforma and included: gender, age at presentation, method of presentation (sporadic vs familial), location of tumour, presence of bilateral/multifocal disease and evidence of malignancy. Molecular genetic testing information was also
collected. For the purposes of this study, identified cases in our UK series and cases identified in the literature were divided into; group A), individuals with both RCC and PPGL/HNPGL and group B), cases where the two tumour classes occurred in two relatives.

4.32 Molecular genetic testing of case series

Some cases referred before 2011 had individual gene testing (e.g. VHL and SDHB) but more recent cases were tested for a panel of up to 10 susceptibility genes (SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, MAX, TMEM127, RET, FH) mostly using a next generation sequencing (NGS) based assay described previously (126).

See methods section, chapter 2 for sequencing methods.

4.33 Histology review

Central renal tumour pathology review was undertaken (when suitable samples were available) by an experienced uropathologist (Dr Anne Warren, Department of Histopathology, Cambridge University Hospitals NHS Trust) and myself. The purpose of histology review was to identify suspicious pathological features, which may be associated with, or predict, non-VHL RAPTAS. The pathologist was blinded to the mutational status and SDHB immunohistochemistry was performed on 4um sections of paraffin embedded tissue using a commercially available SDHB polyclonal rabbit antibody (Sigma Aldrich, United Kingdom) at a dilution of 1:300.

4.34 Tumour sequencing

See methods section, chapter 2.
4.35 Bioinformatics

See methods section, chapter 2.

4.36 Multiple primary tumour score

I applied a previously described multiple tumour score (MTS) to cohort (A) to evaluate if there was a difference in this score between those patient identified with a genetic mutation compared to those without a mutation. This score was designed to predict the likelihood of an inherited cancer syndrome (200).

4.37 Literature review

A full review of the published literature on the genes reported to predispose to PPGL or RCC up to June 2017 was performed. This search was performed and included publications indexed in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) up to June 2017. In addition, the Human gene mutation database (www.hgmd.cf.ac.uk), the Leiden Open Variation Database was interrogated.

4.38 Statistical analysis

Statistical tests were performed using SPSS. Summary statistics include mean and standard deviation for continuous variables and frequency and percentage for categorical variables. A two sample T-test was applied to parametric means and a Mann Whitney test was applied as the non-parametric equivalent test. A Fisher’s exact test was used to calculate the statistical difference between proportions of wild type verses alternate allele reads.
4.4 Results

4.41 Literature Review

A total of 21 kindreds and 39 patients were identified from the literature with a non-VHL RAPTAS phenotype and a germline mutation. Mean age of tumour onset was 36.1 years (17-61 years) (31.8 years (17-47 years) for PC/PGL/HNPGL and 41.4 (19-61 years) years for RCC). The most commonly mutated gene was SDHB (16/21 kindreds) and 44% (7/16) had an exonic deletion (missense in 5/16 and truncating mutations in 4/16). Metastatic RCC was reported in 5 patients with pathogenic SDHB variants one patient with a pathogenic SDHC variant and one patient with a pathogenic SDHD variant (Table 4.2). Three cases had bilateral RCC and one bilateral PC. Metastatic PPGL occurred in one patient. Renal oncocytsoma was described as part of three cases of RAPTAS (two with a SDHB mutation and one with a MAX mutation) (Table 4.2).

In addition to patients with RAPTAS, separate case reports of PC/PGL/HNPGL or renal tumours have been reported in association with the 6 genes described in Table 4.2, as well as with pathogenic variants in FH(66) and SDHA(105) (though no cases of co-existing PC/PGL and RCC in the same patient had been reported in conjunction with a mutation in FH or SDHA). Although there are very rare cases of tuberos sclerosis and neurofibromatosis type 1 with a RCC or phaeochromocytoma respectively, these do not cause diagnostic difficulties because of the syndromic features in such cases and to the best of my knowledge, have not been reported to cause RAPTAS (201) (202).
Table 4.2: Clinical and molecular genetic features of non-VHL RAPTAS cases identified in the literature (*= metastatic disease)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Group A/B</th>
<th>PPGL location and (age)</th>
<th>RCC tumour type and (age)</th>
<th>Sex</th>
<th>Tumour of relative and (age)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHB</td>
<td>c.3G&gt;A</td>
<td>A+B</td>
<td>PGL (25)</td>
<td>Bilateral RCC (25)</td>
<td>M</td>
<td>RCC, brother (23)</td>
<td>(113)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.3G&gt;A</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (23)</td>
<td>M</td>
<td>RCC, PGL, brother (25)</td>
<td>(113)</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 3 deletion</td>
<td>A</td>
<td>HNPGL (30)</td>
<td>Unilateral RCC (36)</td>
<td>M</td>
<td></td>
<td>(113) (103)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.166-170 del CCTCA (p.Pro56TryfsX5)</td>
<td>A</td>
<td>PGL (28)</td>
<td>Unilateral RCC (28)</td>
<td>M</td>
<td></td>
<td>(103)</td>
</tr>
<tr>
<td>SDHB</td>
<td>C.423+1G&gt;A</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC</td>
<td>M</td>
<td>RCC, brother (25)</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 1 deletion</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC* (36)</td>
<td>M</td>
<td>RCC, brother (25)</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 1 deletion</td>
<td>A+B</td>
<td>PC</td>
<td>Unilateral RCC (42)</td>
<td>F</td>
<td>PGL, sister</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>268C&gt;T</td>
<td>A+B</td>
<td>PGL</td>
<td>Unilateral RCC (61)</td>
<td>M</td>
<td>PGL, son</td>
<td>(103)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.286G&gt;A</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (52)*</td>
<td>F</td>
<td>RCC, daughter</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.541-2A&gt;G</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (19)</td>
<td>F</td>
<td>PGL, mother</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.689G&gt;A</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (52)</td>
<td>F</td>
<td>PGL, daughter</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.541-2A&gt;G</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (50)</td>
<td>M</td>
<td>RCC, Brother*</td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 1 deletion</td>
<td>A</td>
<td>PGL (17)</td>
<td>Unilateral renal oncocyteoma</td>
<td>F</td>
<td></td>
<td>(107)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.170A&gt;G</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC* (28)</td>
<td>M</td>
<td>PGL, Mother *</td>
<td>(3)</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.847-50delTCTC</td>
<td>A+B</td>
<td>Unilateral RCC (24)</td>
<td>PGL</td>
<td>M</td>
<td>RCC, PGL, Brother (26)</td>
<td>(3)</td>
</tr>
<tr>
<td>SDHC</td>
<td>c.397C&gt;T</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (53)*</td>
<td>F</td>
<td>RCC, son (40)</td>
<td>(107)</td>
</tr>
<tr>
<td>Gene</td>
<td>Mutation</td>
<td>Segregation</td>
<td>Phenotype</td>
<td>Affected Relative</td>
<td>Relative(s)</td>
<td>Ref(s)</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------------</td>
<td>-------------</td>
<td>-------------------------</td>
<td>------------------</td>
<td>------------------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>SDHC</td>
<td>c.3G&gt;A (p.Met11)</td>
<td>B</td>
<td>HNPGL (46)</td>
<td>Bilateral RCC (48,60)</td>
<td>M Bilateral RCC, mother (48,60)</td>
<td>(203)</td>
<td></td>
</tr>
<tr>
<td>SDHD</td>
<td>c.239G&gt;T (p.Leu80Arg)</td>
<td>A+B</td>
<td>Bilateral HNPGL (17), PGL(28)</td>
<td>Unilateral RCC (45)*</td>
<td>M HNPGL, father, PC brother</td>
<td>(107)</td>
<td></td>
</tr>
<tr>
<td>TMEM127</td>
<td>c.308delG (p.Gly103Alafs)</td>
<td>A</td>
<td>PC (47)</td>
<td>Unilateral RCC (47)</td>
<td>F</td>
<td>(204)</td>
<td></td>
</tr>
<tr>
<td>MAX</td>
<td>Exon 1+2 deletion</td>
<td>A+B</td>
<td>Bilateral PC (45)</td>
<td>Unilateral oncocytoma (45)</td>
<td>M Bilateral PC, brother (28)</td>
<td>(205)</td>
<td></td>
</tr>
</tbody>
</table>
4.42 Case series demographics and clinical features

33 individuals (16 male and 17 females) with PC/PGL/HNPGL and/or a renal tumour from 22 kindreds without clinical or molecular evidence of VHL disease met our criteria for the diagnosis of non-VHL RAPTAS. This cohort was subdivided into two groups: (A) Multiple tumour patients (n=12 probands) and (B) familial non-VHL RAPTAS cases with RCC or PC/PGL/HNPGL in first degree relatives (n=21 patients, 10 probands).

Clinical Features of Group A: Multiple Tumour non-VHL RAPTAS Cases

Twelve patients with a diagnosis of PC/PGL/HNPGL and a renal tumour were identified. The clinical details are summarised in Table 4.3. Seven cases had synchronous tumours and five metachronous. Mean age at diagnosis of first tumour was 55.3 years (SD 19.4, range 10-76 years). 4/5 metachronous cases, presented with PC/PGL/HNPGL and one patient was initially diagnosed with RCC. In most cases a unilateral PC was present (75%, 9/12 patients) but there were two cases (16.6%) with HNPGL and one with an abdominal PGL. Most renal tumours were RCC (91.7%, 11/12 patients) but a renal oncocytoma was present in a single patient. One group ‘A’ patient had been diagnosed with breast carcinoma but no additional tumours such as GIST, thyroid, or pituitary tumours were identified in group ‘A’ or group ‘B’ patients (Table 4.3 and Table 4.4).
Table 4.3: Clinical features and genetic features of RAPTAS patients with multiple tumours (Group A)

<table>
<thead>
<tr>
<th>Proband #</th>
<th>Age at diagnosis of first tumour (second tumour)</th>
<th>Phenotype</th>
<th>Metastatic disease</th>
<th>Germline genetic analysis</th>
<th>Histology reviewed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63 (63)</td>
<td>Unilateral renal oncocytooma, Unilateral PC</td>
<td>No</td>
<td>No detectable variant in <em>SDHA, SDHB/SDHC/SDHD, SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>76 (76)</td>
<td>Unilateral RCC, Unilateral PC</td>
<td>No</td>
<td>No detectable variant in <em>SDHA, SDHB/SDHC/SDHD, SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>56 (56)</td>
<td>Unilateral RCC, Unilateral PC, Breast carcinoma</td>
<td>No</td>
<td>No detectable variant in <em>SDHA, SDHB/SDHC/SDHD, SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>62 (64)</td>
<td>Unilateral PC, Multifocal RCC</td>
<td>Yes (RCC)</td>
<td>No detectable variant in <em>SDHA, SDHB/SDHC/SDHD, SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>68 (68)</td>
<td>Unilateral PC, Unilateral RCC</td>
<td>No</td>
<td>No detectable variant in <em>SDHB or VHL</em></td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>41 (41)</td>
<td>Carotid body PGL, Unilateral RCC</td>
<td>No</td>
<td>Variant of uncertain significance in <em>SDHD (c.34G&gt;A p.Gly12Ser)</em> Tested for SDHB/C/D and VHL, No</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>60 (60)</td>
<td>Unilateral RCC, Unilateral PC</td>
<td>No</td>
<td>No detectable variant in <em>SDHB or VHL</em></td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>10 (26)</td>
<td>Abdominal PGL, Unilateral RCC</td>
<td>No</td>
<td><em>SDHB c.141G&gt;A (p.TRP47</em>) Tested for VHL and SDHB</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>62 (63)</td>
<td>Unilateral PC, Unilateral RCC</td>
<td>No</td>
<td><em>SDHB c.268C&gt;T (p.Arg90</em>) Tested for SDHB, VHL</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>43 (43)</td>
<td>Unilateral RCC</td>
<td>No</td>
<td>MAX c.97C&gt;T (p. Arg33*) (Tested for SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX and FH).</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>53 (62)</td>
<td>Unilateral PCC</td>
<td>No</td>
<td>TMEM127 c.117_120delGTCT (Tested for SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX and FH.)</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>34 (39)</td>
<td>Carotid body HNPGL</td>
<td>Yes (RCC)</td>
<td>SDHB c.79C&gt;T (P.Arg27*). Tested for SDHB, SDHC, SDHD and VHL</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Clinical Features of Group B: Familial non-VHL RAPTAS Cases**

10 kindreds were identified containing two or more first degree relatives (FDRs) with PC/PGL/HNPGL and a renal tumour. Information including phenotype, genotype and demographic information was available on 10 probands (6 female, 4 male) referred for genetic testing and basic demographic/phenotype information was available on the 11 affected FDRs (2 females, 9 males) (see Table 4.4). Mean age at presentation of the probands was 56.6 years (SD 17.3, range 27-77 years) and mean age at tumour diagnosis in 11 affected FDRs was 52.3 years (SD 16.3, range 13-65 years). In eight kindreds the proband presented with a PC/PGL/HNPGL (3 with a PC, 3 HNPGL 2 abdominal PGL) and in two cases had malignant paragangliomas (a HNPGL and an abdominal PGL) (see Table 4.4). Two probands presented with RCC and a renal oncocytoma. Most probands in group (B) had one affected relative but one proband had two affected relatives (2 brothers, both with RCC).
**Table 4.4:** Clinical and molecular features of RAPTAS kindreds with PPGL and a renal tumour in two first degree relatives (Group B) (*= metastatic disease)

<table>
<thead>
<tr>
<th>Proband #</th>
<th>Age at diagnosis</th>
<th>Phenotype of Proband</th>
<th>Pathogenic genetic variant identified in proband</th>
<th>Relative affected</th>
<th>Phenotype of relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>56</td>
<td>Renal oncocytoma</td>
<td><em>Pathogenic SDHB splice site intron variant IVS1 + 1 G&gt;T</em></td>
<td>Daughter (13)</td>
<td>Unilateral PC</td>
</tr>
<tr>
<td>14</td>
<td>50</td>
<td>HNPGL*</td>
<td>No detectable variant in <em>SDHB</em> or <em>VHL</em></td>
<td>Father (58)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>15</td>
<td>77</td>
<td>Unilateral PC</td>
<td>No detectable variant in <em>SDHA, SDHB/SDHC/SDHD,SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>Daughter (51)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
<td>HNPGL</td>
<td>No detectable variant in <em>SDHA, SDHB/SDHC/SDHD,SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>Brother (54)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>17</td>
<td>57</td>
<td>Abdominal PGL*</td>
<td><em>SDHB</em> c.166-170delCCTCA (p.Pro56Tyrfs5X)*</td>
<td>Brother (57)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>18</td>
<td>67</td>
<td>Abdominal PGL</td>
<td>No detectable variant in <em>SDHB</em> or <em>VHL</em></td>
<td>Brother (52)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Unilateral PC</td>
<td>No detectable variant in <em>SDHA, SDHB, SDHC, SDHD,SDHAF2, MAX, TMEM127, FH, VHL</em></td>
<td>Father (65)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>Unilateral RCC</td>
<td><em>SDHB</em> c.380T&gt;G (p.Ile127Ser)*</td>
<td>Brother (64)</td>
<td>Unilateral PC</td>
</tr>
<tr>
<td>21</td>
<td>60</td>
<td>Unilateral PC</td>
<td>No detectable variant in <em>SDHB</em> or <em>VHL</em></td>
<td>2 brothers (50,63)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>22</td>
<td>27</td>
<td>Unilateral PC</td>
<td>No detectable variant in <em>SDHB</em> or <em>VHL</em></td>
<td>Father (49)</td>
<td>Unilateral RCC</td>
</tr>
</tbody>
</table>
4.43 Molecular Genetics Analysis of the non-VHL RAPTAS case series

Molecular genetic analysis was performed on all 22 probands from Groups A and B. All cases were tested for pathogenic germline variants in \textit{VHL}, and \textit{SDHB} and 8/12 (67\%) of probands from group A and 6/10 (60\%) of probands from cohort B were also tested for pathogenic variants in \textit{SDHA}, \textit{SDHC}, \textit{SDHD}, \textit{SDHAF2}, \textit{FH}, \textit{MAX} and \textit{TMEM127}. A pathogenic germline \textit{SDHB} variant (four truncating mutations and a splice site mutation) was detected in 6/22 (27.3\%) probands (3 from Group A and 3 from Group B). Family testing was possible in 2 of 3 Group B kindreds and in both cases the affected relative harboured the pathogenic \textit{SDHB} variant detected in the proband.

One proband was diagnosed with a variant in \textit{SDHD} (c.34G>A, p.Gly12Ser), that was not considered pathogenic and did not prompt family screening. One proband presenting with RCC and unilateral PC aged 43 years had a truncating variant in the \textit{MAX} gene (Table 4.3, case #10). This NGS result was confirmed by Sanger sequencing (Figure 4.1). Another proband from Group (A) was found to have a truncating mutation in \textit{TMEM127} (Table 4.3, case #11).

No statistically significant correlation was identified for younger age at first tumour diagnosis, PGL, renal oncocytoma or malignant PGL and the identification of a pathogenic germline variant (p>0.05 for all associations). The mean multiple tumour score value in Group A patients with a mutation was 3.6 compared to 1.8 in those without a mutation (P=0.09).
4.44 Histology review

Archival tumour samples were available for 4 patients from cohort A (RCC samples from probands #2, #3, #11 and #12 and a PC from #2) and histology review and SDHB immunostaining was performed (Figure 4.2 and 4.3). One case (proband #12) had histological features of an SDH-deficient RCC and had corresponding loss of SDHB protein expression on immunohistochemistry (Figure 4.3). A PC from case #2 showed loss of SDHB expression on immunohistochemistry but SDHB expression in the RCC was preserved and histology showed a papillary RCC subtype (Figure 4.4).

4.45: Tumour sequencing

Analysis of DNA extracted from the phaeochromocytoma and renal cell carcinoma from case #10 with the germline truncating variant in the MAX gene (c.97C>T p. Arg33*), revealed loss of heterozygosity (See Figure 4.5), with higher reads in the mutant allele identified in the phaeochromocytoma (reads wild type/mutant: 77/151, depth 228) and RCC (reads: 60/179, depth 239). compared to the germline (157/157, depth 314) (germlineversus.PC P=0.0002; germline versus RCC P<0.0001 (Fisher’s exact test). No additional somatic variants were identified in other RAPTAS-related genes (VHL, SDHA, SDHB, SDHC, SDHD, FH, TMEM127) in either tumour from case #10.

Loss of SDHB immunostaining in the PC from case #2 prompted additional sequencing of tumour tissue from the PC and RCC as germline testing did not reveal a germline mutation in SDHx or VHL (Table 1). No somatic variant in SDHA/SDHB/SDHC/SDHD was identified in either tumour but a somatic variant (not present in the germline) in VHL (c.245G>T p.Arg82Leu) was identified in the PC tumour but not the RCC from case #2 (Figure 4.6)
**Figure 4.1:** DNA sequencing chromatogram from the germline DNA of case #11 obtained confirms the presence of a truncating variant (c.97C>T (p. Arg33*)) in the MAX gene as highlighted by the arrow in this figure by Sanger sequencing methods.

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**Figure 4.2 a)** Demonstrates the H+E stained compact architecture and overall histological features consistent with a clear cell RCC from proband # 11 with a pathogenic *TMEM127* variant, which demonstrated preserved SDHB immunostaining (4.2b). Figure 4.2c) demonstrates a chromophobe RCC tumour from proband # 3, with no detectable germline variant (H+E staining X 200hpf). Figure 4.2d) again shows positive SDHB immunostaining of the chromophobe RCC tumour. Figures courtesy of Dr Anne Warren.
**Figure 4.3a)** Displays the H+E stained histological appearance of the SDHB deficient RCC from proband #12, with evidence of intracytoplasmic vacuoles marked by the black arrow and loss of SDHB protein expression on immunostaining of the RCC tumour (3.3b) from proband #12 in the lower part of the image with SDHB staining present in the adjacent normal renal tissue visible in the upper image.

**Figure 4.4a)** Demonstrates the histological appearances of a renal papillary carcinoma from proband #2 (H+E staining X 200hpf) and preserved SDHB expression on immunostaining in this tumour is displayed in 4.4b). Figure 4.4c) shows a phaeochromocytoma tumour from proband #2 and image 4.4d) demonstrates loss of SDHB immunostaining in the phaeochromocytoma, the black arrow points to an area of normal adrenal tissue with preserved SDHB protein expression.
**Figure 4.5:** This figure illustrates preferential loss of the wild type allele in both phaeochromocytoma PC and RCC tumours from patient #10 with the identified pathogenic germline variant in MAX (c.97C>T p. Arg33*).

**Figure 4.6:** This figure displays evidence of a somatic VHL mutation (c.245G>T p Arg82Leu) in the PCC tumour from case two with no evidence of this mutation in germline or RCC tumour in case 2 on analysis of BAM files on IGV.
4.5: Discussion

This large case series and literature review demonstrated that non-VHL RAPTAS is genetically heterogeneous. RAPTAS may be caused by pathogenic germline variants in six genes (VHL, SDHB, SDHC, SDHD, TMEM127 and MAX) and two further genes, FH and SDHA have each been reported to predispose to both groups of tumours (PPGL and renal tumours) and may yet be described as a cause of RAPTAS. In both the literature review and case series, SDHB variants were the most common identified cause of non-VHL RAPTAS. Less frequently RAPTAS was associated with pathogenic variants in other SDHx genes, TMEM127(204) and MAX (205).

A limitation of this study was that all cases had not been tested for variants in the rarer RAPTAS genes (SDHC, TMEM127 and MAX) and a limitation of the literature review is probable bias against reports of RAPTAS without an identified genetic diagnosis. Nevertheless, this study found that there is a substantial group of RAPTAS patients without an identified germline mutation suggesting that further RAPTAS genes are still to be identified.

Recently Kopershoek et al described a germline MAX variant (a large complex genomic alteration encompassing the intragenic and promoter regions of MAX and FUT8) in a patient with renal oncocytoma, bilateral phaeochromocytoma and erythrocytosis and two siblings with bilateral phaeochromocytoma (205). In this study the first association of RCC with a pathogenic germline MAX variant (c.97C>T p. Arg33*) was identified. Tumour sequencing demonstrated evidence of preferential loss of the wild type allele in both tumours (PC and RCC) similar to previously reported cases of MAX-related tumours(206). This finding expands the phenotype associated with pathogenic MAX variants and raises the intriguing possibility that MAX may be a candidate gene for inherited RCC, similar to how SDHB was
eventually implicated in hereditary RCC. *SDHB* variants were originally described in association with PC/PGL/HNPGL (60), then with RAPTAS (3) and then familial RCC (108) only phenotypes. Though variants in all RAPTAS genes are inherited in autosomal dominant manner, variants in *MAX* and *SDHD* show a parent-of-origin dependent tumourigenesis and tumours occur almost exclusively following paternal transmission of the mutation. Hence the clinical management and genetic counselling of RAPTAS kindreds with pathogenic *SDHD* and *MAX* variants will differ from those with mutations in other RAPTAS genes.

In this study, a second case of a patient with RAPTAS due to a pathogenic variant in *TMEM127* was identified. The first report was in a 47 year old lady with multifocal unilateral phaeochromocytoma and a unilateral (clear cell) RCC (204). A germline deletion variant in *TMEM127* (c.308delG) and an additional germline variant in *SDHB* (159_*184delins25) was identified in this patient but SDHB immunohistochemistry showed preservation of SDHB expression in both tumours. Histology of the RCC in RAPTAS patient #11 with a *TMEM127* mutation demonstrated a clear cell RCC. Though this is the most common type of RCC, the four additional reported cases of TMEM127 associated RCC were all clear cell variant RCC (199).

### 4.51 Role of clinical features in suggesting specific genes

In genetically heterogeneous conditions it is helpful if specific clinical features can guide genetic testing. Clear cell RCC, PC (less often PGL and rarely HNPGL) and retinal and central nervous system haemangioblastomas (or the presence of pancreatic or renal cysts) should prompt genetic testing for pathogenic VHL variants. The occurrence of HNPGL, abdominal PGL and malignant PPGL or the co-occurrence of wild-type GIST tumours, suggests a possible pathogenic *SDHx* variant. Adrenal PC is more common in VHL disease.
whereas extra-adrenal PGL is more commonly associated with \textit{SDH}x variants. However the secretory pattern (predominantly noradrenergic) is similar with \textit{VHL} and \textit{SDH}x and there are similar features on PET CT with tracers such as 18-fluorodeoxyglucose (207).

Indicators of an inherited cancer predisposition syndrome include the occurrence of uncommon/rare tumours in the same individual, related tumour types in close relatives, early age at diagnosis and the presence of multicentric disease. In patients with RCC, genetic investigation should be considered in sporadic cases aged <45 years (194). Though individuals identified by the literature review with non-VHL RAPTAS and a pathogenic germline variant had relatively young onset of PPGL (mean age 31.8 years) and RCC (mean 41.4 years), in the case series there was no clear relationship between age at tumour diagnosis and presence/absence of a mutation. Though the difference in the multiple tumour score between mutation positive and negative cases did not reach statistical significance further studies are required to determine the utility of this score in Group A RAPTAS cases. Though RAPTAS might in some cases arise coincidentally, it was notable that for two \textit{SDHB} variant positive cases in this series (probands #9 and #18) age at tumour diagnosis was 60 years or older. Therefore, this would suggest that either all cases of RAPTAS should undergo molecular investigation or the age-at-tumour-diagnosis cut-off for not pursuing genetic testing should not be less than 70 years.
4.52: Role of histology in suggesting specific genes in RAPTAS

It is now evident that histopathological features may be used to prioritise likely genetic causes of hereditary RCC (194): pathogenic germline *VHL* variants are invariably associated with clear cell RCC (194) and a unique morphology consisting of solid architecture, distinctive intracytoplasmic inclusions and intra tumour mast cells is characteristic of SDHB-deficient RCC (104) (See Figure 4.3a). Given that pathogenic *SDHB* gene variants were the most common gene implicated in RAPTAS in this study, immunohistochemistry is a useful diagnostic adjunct as SDHB-deficient tumours show negative immunoreactivity. Interestingly proband #2 had evidence of SDH deficiency on SDHB immunostaining of the PC (Figure 4.4d) but immunostaining showed preserved SDHB expression in the RCC tumour (Figure 4.4b). Sequencing of both the PC and RCC tumours in this case (#2) revealed a driver somatic variant in *VHL* (c.245G>T p Arg82Leu) in the PC but not the RCC with no evidence of a variant in *SDHA/SDHB/SDHC/SDHD* genes. False positive results using SDHB immunohistochemistry (as apparently occurred in this case) have been reported for patients with pathogenic germline *VHL* variants (7). A potential alternative explanation for the discrepant SDHB immunohistochemistry results in case #2, is that the first hit is an undetected germline *VHL* variant (e.g. intronic mutation or a copy number alteration) and that the somatic *VHL* missense variant in the PCC was the “second hit”. However, the RCC histology was a papillary, whereas renal tumours in VHL disease are clear cell. Nevertheless it is important to consider that pathogenic *VHL* variants can lead to false positive results on SDHB IH, therefore it would be best to recommend that those patients with RAPTAS, without a detectable germline variant in *SDHx*, but with loss of SDHB immunoreexpression on tumour studies, undergo genetic screening for pathogenic *VHL* variants.
4.53 Investigation of potential RAPTAS patients

In study group ‘A’ patients, a third presented with PPGL and were subsequently diagnosed with a RCC. The longest time interval between the presenting tumour (abdominal PGL) and diagnosis of RCC was 16 years (proband #8). For the other three patients, the mean interval between the first tumour and RCC was 4 years (median 2 years, range 1-9 years). Recently published European guidelines recommend a 10 year follow up for patients with sporadic PC and life-long follow up for patients with PPGL or those patients with a confirmed genetic predisposition(208). The recommendations for follow up include biochemical and radiological surveillance which would include abdominal imaging capable of detecting renal tumours. Data from this study suggest that this surveillance protocol will facilitate the detection of patients with RAPTAS.

Patients meeting clinical criteria for RAPTAS should be referred for genetic testing. If gene panel testing is not available/undertaken then single gene testing should be prioritised as suggested in Figure 4.7. It is important that $SDHB$ variant analysis includes analysis for exonic deletions/duplications as the literature review revealed a higher than expected proportion of deletions in $SDHB$-associated RAPTAS. Positive cases should receive appropriate follow-up and surveillance. The clinical benefits of identifying germline genetic variants in patients with non-VHL RAPTAS also include family screening and in the future genetic classification may facilitate a personalised treatment approach, as has been the case for hereditary RCC (195).
Figure 4.7: Flow chart of the recommended genetic evaluation of potential RAPTAS kindreds
4.6 Conclusion

The term RAPTAS (rather than inherited phaeochromocytoma-renal cell carcinoma) emphasises that many cases may be sporadic with no family history and that not all cases may have a genetic origin. This study is the largest study to date of non-VHL RAPTAS and has enabled the development of guidelines for genetic testing in suspected RAPTAS and for clinical diagnostic criteria that include both sympathetic and parasympathetic PPGL and both malignant (RCC) and benign (oncocytoma) renal tumours in the criteria. Application of whole exome and whole genome sequencing to undiagnosed RAPTAS cases in the future may provide further insights into the molecular mechanisms of this association and improve the therapeutic management of these cases.
Chapter 5

Investigating the somatic mutation landscape of hereditary and sporadic phaeochromocytoma and paraganglioma

DECLARATION: Figures 5.1 and 5.2 have been adapted and included in a chapter entitled: “Genetics of phaeochromocytomas, paragangliomas and neuroblastoma”, which is being considered for publication in the Oxford Medical handbook of Endocrinology and Diabetes.
Chapter 5: Investigating the somatic mutation landscape of hereditary and sporadic phaeochromocytoma and paraganglioma (PPGL)

5.1 Introduction

5.11: Somatic tumor sequencing

5.12: Signaling pathways implicated in PPGL

5.2 Aims

5.3 Methods

5.31: Tumour samples and analysis

5.32: Next generation tumour sequencing

5.33: Bioinformatics analysis

5.34: Variant filtering

5.35: Primer design

5.36: Sanger sequencing

5.37: Measuring Phenylethanolamine N-methyltransferase (PNMT) gene expression using real-time polymerase chain reaction (RT-PCR)

5.38: Statistical analysis

5.39: Literature Review

5.4 Results

5.41: Cohort demographics and clinical features of discovery cohort

5.42: Technical assessment of next generation sequencing assay

5.43: Tumour sequencing of inherited PPGL cases

5.44: Somatic mutation landscape of discovery and validation tumour cohort

5.45: Relationship between clinical phenotype and somatic driver variants in discovery cohort
5.46: Molecular pathways affected by identified pathogenic somatic variants

5.5 Discussion

5.51: Translational utility of somatic profiling in PPGL

5.52: The role of next generation sequencing assays in somatic tumour profiling

5.6 Conclusion
5.1 Introduction

Phaeochromocytoma and paraganglioma (PPGL) are now considered to be the most heritable tumours and over the past two decades more than a dozen PPGL susceptibility genes have been identified (198). In comparison to the advances made in germline susceptibility gene discovery, relatively little is known about the somatic mutational landscape of PPGL. Therefore identifying somatic driver variants in PPGL has the potential to have additional diagnostic, prognostic and therapeutic utility and could also serve to ease anxiety about a potential familial predisposition, if a somatic driver variant is discovered in a patient with no identified pathogenic germline variant. Furthermore, tumour sequencing has become more amenable in the era of next generation sequencing, which offers a faster, cheaper and higher throughput option to the conventional method of Sanger sequencing (209). Custom next generation sequencing panels for tumour (210) have followed in the successful path of germline targeted assays (126) and testing can be applied to paraffin embedded tissues as well as fresh frozen samples (210).

5.11 Somatic tumor sequencing

It is estimated that 30-40% of sporadic PPGL will harbor a driver somatic variant (211)(212), and somatic variants have been identified in paediatric cases, metastatic cases (211) and young onset adult cases of PPGL (211)(212). In contrast to the genotype-phenotype correlation which exists for PPGL and known germline variants, with extra adrenal and bilateral tumours having a greater risk of harbouring a germline variant (198), a recent study investigating the tumour genotype of 99 PPGL from patients with no pathogenic germline variant in RET, VHL, HRAS, EPAS1, MAX or SDHB, identified that up to half of sporadic phaeochromocytoma (PC) had a somatic mutation compared to a third of paraganglioma
(PGL) tumours (212). This study recommended that germline mutational testing should take priority for those patients with extra adrenal PGL and that somatic sequencing should be prioritised for those patients with sporadic PC and malignant PPGL.

Studies to date have demonstrated that HRAS and NF1 are the most frequently mutated genes in sporadic PC. In particular pathogenic HRAS variants have been described in sporadic tumours exclusively with no report of a pathogenic HRAS variant occurring in a patient with a co-existing pathogenic germline variant (209) (213) and the prevalence of somatic HRAS variants has been estimated at 7-15% (212)(213)(214). Almost all RAS variants described in cancer and indeed in PPGL to date occur in 1 of 3 highly conserved codons (glycine 12, glycine 13, or glutamine 61) resulting in an activated RAS protein (209)(212)(213)(214). The clinical phenotype described in association with somatic HRAS variants includes adrenergic adrenal PC tumours, with rare cases of PGL or malignant tumours (209)(212)(213)(214).

Somatic NF1 variants in PPGL have been reported at a prevalence of 25-40% (215)(216) and as such, NF1 is currently recognized as the foremost mutated gene in sporadic PPGL. The phenotype associated with somatic NF1 mutations is similar to that of HRAS, namely sporadic benign adrenergic PC and in one study, most cases of reported somatic NF1 mutations demonstrated loss of heterozygosity at the NF1 locus in the tumour (215).

Somatic variants in RET, VHL and EPAS1 complete the list of the more commonly implicated genes in sporadic PPGL. Somatic pathogenic RET variants occur at a frequency of 5-15% (209)(211)(212)(217) and again are more commonly associated with adrenergic sporadic PC. In contrast to the phenotype predicted by somatic RET variants in medullary thyroid carcinoma, which predicts a higher risk of malignant potential, somatic RET variants in PPGL have not been associated with a more aggressive tumour behavior (217).
Somatic variants in VHL and EPAS1 have been less commonly reported in sporadic PPGL and are described in association with a predominately noradrenergic secretory phenotype. Pathogenic somatic VHL variants are associated with sporadic PC (209)(211)(212), while pathogenic EPAS1 variants have also been implicated in sporadic PGL (212) as well as PC (71).

A strong genotype-phenotype correlation exists for germline missense variants in VHL and the occurrence of PC compared to truncating or deleterious VHL variants (218), which are more commonly associated with renal cell carcinoma (219). Similarly the vast majority of somatic VHL variants reported to date are missense variants (211) (220).

Importantly EPAS1 variants have been associated with mosaicism (221) and therefore if an EPAS1 variant is identified in an apparently sporadic tumour, it is recommended that efforts are made to further clarify the presence of the variants in other tissue types, to inform the extent of disease and the appropriate surveillance approach for the patient and relatives (221).

Less common genes implicated in sporadic PPGL includes the ATRX gene, a large gene located on the X chromosome, which serves a crucial regulatory role in chromosome stability. Germline variants in ATRX are implicated in a severe childhood onset neurodevelopmental syndrome, while somatic variants in ATRX have been described in a number of tumours including sporadic PPGL (222). ATRX variants in sporadic PPGL have demonstrated a potential role as driver mutations in clinically aggressive tumours (222).

Despite the established role of the citric acid cycle genes; SDHA/SDHB/SDHC/SDHD and FH in hereditary PPGL (198), pathogenic somatic variants in these genes are rarely reported (209) (212) (223). Similarly the contribution at a somatic level of other novel PPGL predisposition genes such as TMEM127 and MAX, is very rare. (209)(212)(206).
5.12 Signalling pathways implicated in PPGL

Investigating the genomic profile of PPGL has facilitated a better understanding of the molecular pathways implicated in the development of these tumours and presents an opportunity to explore and develop new potential therapeutic targets. Understanding the molecular drivers in inherited PC and PGL has enabled further interrogation of the associated transcription profiles and segregation of inherited PPGL into two categories. Category 1 is referred to as the ‘pseudohypoxic cluster’ based on the up-regulation of hypoxia signalling pathways (224) and ‘cluster 2’ is characterised by an upregulation of kinase signalling pathways (79). Tumours caused by pathogenic variants in the VHL, FH, SDHx and EPAS1 genes fall into the pseudohypoxic cluster (198). Cluster two genes include RET, NF1, TMEM127, MAX, HRAS, and variants in these genes activate the kinase pathways including the i) PI3K/AKT, ii) RAS/RAF/ERK, and iii) mTORC1 pathways.

More recently Fishbein and colleagues proposed an extended molecular classification of PPGL and suggested two further categories namely a cortical admixture subtype and a Wnt-altered subtype (225). The Wnt-altered subtype is characterised by overexpressed genes in the Wnt and Hedgehog signalling pathways and perturbations in this pathway were identified exclusively in sporadic phaeochromocytomas, driven by newly identified somatic variants in CSDE1 and MAML3 fusion genes (225).

Reviewing the somatic variant landscape of PPGL in isolation, the most common molecular pathway implicated is the RAS/RAF/ERK pathway (226). This pathway is commonly implicated in tumourigenesis (227) and consists of a series of serine-threonine kinases functional in a sequential pathway to regulate cell cycle factors such as cyclin D and proto-oncogenes such as c-MYC (228). Somatic variants in RET and NF1 as well as HRAS, have been implicated in RAS activation (229). Furthermore the RAS/RAF/ERK pathway interacts closely with the PIK3/AKT pathway such that RAS and PI3K can activate each other (230).
and inhibition of mTORC1 signals a PI3K dependent feedback loop, which in turn activates both AKT and ERK (231) (Figure 5.1). This activation of AKT and ERK triggered by mTORC1 inhibition might explain the disappointing clinical outcomes noted in studies using the mTOR inhibitor everolimus in patients with malignant PPGL (232), as this mechanism of resistance to mTORC1 inhibition has been described in other neuroendocrine tumours (233). Loss of function variants in the SDHx genes leads to the accumulation of the oncometabolite succinate, and recently rare pathogenic somatic variants in another citric acid cycle regulatory gene IDH1 have been reported in PPGL (234). Variants in IDH1 result in accumulation of the oncometabolite; 2-hydroxyglutarate (2HG) and accumulation of both succinate and 2HG act to inhibit alpha ketoglutarate dependant di-oxygeenase enzymes, promoting stabilisation of hypoxia inducible factor (HIF) complex (79) and inhibiting histone and DNA demethylation enzymes, resulting in a hypermethylation phenotype (83). Similarly, in the presence of an altered VHL protein, the HIF alpha subunit does not undergo VHL mediated proteolytic degradation but rather translocates to the nucleus, where it dimerizes with HIF-beta to form transcriptionally active HIF complex (235) (Figure 5.2). Stabilisation of HIF facilitates the transcriptional upregulation of target genes including glycolytic genes and vascular epidermal growth factor (VEGF) (236).

Sunitinib is a receptor tyrosine kinase inhibitor that inhibits VEGF-R and has demonstrated both anti-angiogenic and antitumor activity in PPGL (237), however evidence to date would also suggest a tendency for therapeutic escape with Sunitinib (238) and evidence from the randomised, double blinded phase 2 multi-centre study; FIRSTMAPP (ClinicalTrials.gov Identifier: NCT01371201), is eagerly awaited.

Furthermore enrolment is currently underway for another phase 2 clinical trial, using a second generation DNA Methyl Transferase Inhibitor, Guadecitabine (SGI-110) (ClinicalTrials.gov Identifier: NCT03165721), for patients with SDHx or fumarate hydratase
(FH) mutated PPGL, due to the hypermethylation phenotype promoted by mutations in these citric acid cycle genes (83).

Finally a recent pre-clinical study has demonstrated efficacy of a small molecule HIF-2α inhibitor (PT2399) in mouse models of primary and metastatic pVHL defective clear cell renal cell carcinoma (239) and therefore offers promise for PPGL and other tumours harbouring EPAS1 as well as germline and somatic variants in other cluster 1 genes.

5.2 Aims

1. To further investigate the role of pathogenic somatic variants in PPGL using a next generation sequencing strategy to analyse "mutation hotspots" in 68 human cancer genes

2. To elucidate the potential clinical utility of somatic variant testing in clinical practice.
**Figure 5.1:** Cluster 2 kinase pathway interactions. Adapted from (231)

**Figure 5.2:** Hypoxia signalling pathways. Adapted from (79)
5.3 Methods

5.31: Tumour samples and analysis

i) Discovery cohort

A total of 32 PPGL tumour and matched germline DNA samples from 32 patients (31 primary, 1 metastatic tumour) were collected after informed patient consent. The samples analyzed included 12 fresh frozen (FF) and 20 paraffin embedded (FFPE) tumour samples.

ii) Validation cohort

38 PPGL tumour (30 FF, 8 FFPE) samples were included in the validation cohort. Matched germline DNA was available on 15/38 (39%) samples and extensive clinical information (other than tumour type and malignancy) was not available on the validation cohort of samples.

5.32: Next generation tumour sequencing

See methods section, chapter 2.

5.33: Targeted sequencing

High quality targeted sequencing using the SeqCap EZ HyperCap workflow (Roche Sequencing Solutions, Inc.) was carried out by colleagues in the Cancer and Molecular Diagnostics Laboratory in Cancer Resarch UK Cambridge. Three tumour samples from cases; #19, #32 and #69 were selected for targeted sequencing as an alternative orthogonal sequencing validation method to Sanger sequencing. 200ng of DNA from these samples was
fragmented individually to 200-300bp by ultrasonication using Covaris M220 focused ultrasonicator. The samples was then taken directly into end repair and A-tailing using a combined enzymatic reaction following Roche SeqCap EZ HyperPrep workflow. Illumina adapters with unique barcodes for the samples were then ligated to each library, before carrying out a bead based clean up and size-selection to select for fragments 240-400bp. The samples was amplified using KAPA HiFi HotStart ReadyMix. The completed library was checked and quantified using D5000 TapeStation kit and Qubit high sensitivity DNA kit respectively. The samples were then multiplexed into a single hybridization reaction with a custom Roche SeqCap EZ capture panel designed to capture the coding regions of 350 genes following the SeqCap EZ HyperPrep protocol. Recovery of the captured samples was carried out using the Roche SeqCap Hybridization and Wash Kit, followed by a second round of amplification. After a final bead based clean-up step, the library proceeded to sequencing.

5.34: Bioinformatics analysis

See methods section, chapter 2.

5.35: Variant filtering

Variant filtering for somatic variants was performed independently. Synonymous variants and non-coding variants were removed. Variants were removed if the variant allele frequency was <1% or the minor allele frequency (MAF) greater than 0.1% in EVS6500 and/or 1000 Genomes. All variants with a read depth less than two standard deviations below the mean coverage (<351 reads) were filtered out. Variants in the intronic and intergenic regions,
synonymous variants, variants which failed the ‘artefact-in-normal’ and ‘base quality’ (minimum base quality below 20) filters, were also discarded. Finally, variants that were classified as ‘benign’ or ‘likely benign’ on the Catalogue of Somatic Mutations in Cancer (COSMIC) (https://cancer.sanger.ac.uk/cosmic) or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) were removed. For those tumour samples without a matched germline, further variant filtering was performed if a common germline variant or single nucleotide polymorphisms was identified. For the purpose of this study, a somatic variant was defined as a potential driver variant if the variant allele frequency was >5%.

5.36 Primer design

Primers were designed in order to validate by Sanger sequencing those potential somatic driver variants detected by the next generation sequencing assay. Only pathogenic somatic variants with a variant allele frequency greater than 15% were validated using Sanger sequencing as a VAF<15% was deemed to be below the threshold of detection for Sanger sequencing. Primers were designed for the individual variants with the aid of the open access software programme Genome Complier (http://www.genomcompiler.com). A full list of the designed primers employed are displayed in Table 5.1.
Table 5.1: Primer sequences for Sanger sequencing of potential pathogenic somatic variants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRAS c.182A&gt;C (p.Gln61Pro)</td>
<td>F:GAAGGTTCTGAGGGGTTCC R:GTGCAGGGGTGGCTCC</td>
<td>63</td>
</tr>
<tr>
<td>VHL c.482G&gt;A (p.Arg161Gln)</td>
<td>F:CCATAGGGGCCCAGACAAACA R:GGCGAGGGTCTITTGTC</td>
<td>63</td>
</tr>
<tr>
<td>SDHB c.423+1G&gt;A</td>
<td>F:CCACTGGATATTTTTCTTTTCTTAGATGTGGCC R:CCAGCCTCTTGGAAAGAACCACAAGTAT</td>
<td>63</td>
</tr>
<tr>
<td>FBXW7</td>
<td>F:TTAAGACAAAAACGCTATGAC R:TCACTTTCCCTTCTACCA</td>
<td>63</td>
</tr>
<tr>
<td>VHL c.245G&gt;T (p.Arg82Leu)</td>
<td>F:CGCGAAGACTACGGAGGT R:GGGTAGCTGCTATGTCG</td>
<td>63</td>
</tr>
<tr>
<td>IDH1 c.394C&gt;T (p.Arg132Cys)</td>
<td>F:GGTGGTACTAGACAGGCTTC R:GGTGGTACTAGACAGGCTTC</td>
<td>63</td>
</tr>
<tr>
<td>SMO c.1576delT (p.Phe526fs)</td>
<td>F:CTCAGGCTATGAGGCTTC R:GGGTTAGCTGCTATGTCG</td>
<td>63</td>
</tr>
<tr>
<td>SDHA c.1270G&gt;T (p.Glu424X)</td>
<td>F:ACCTGGACATTTCACCTGAA R:GAAGGTTGAGGTGTTGAAGA</td>
<td>63</td>
</tr>
<tr>
<td>VHL c.413_414delCA p.Pro138fs</td>
<td>F:TTAACACCTTGGCTTTGTC R:GGCAAAAATTGAGAACTGGG</td>
<td>63</td>
</tr>
<tr>
<td>NF1 c.2927_2933delCTGAAGG (p.Thr976fs)</td>
<td>F:TCTGTATGCTTTATTTGCTCTC R:TTCTGGCTACCTTACTGTC</td>
<td>63</td>
</tr>
<tr>
<td>NF1 c.2098delA (p.Thr700fs)</td>
<td>F:CACACACACAGTTATTTGCA R:CTTTAGGCAGACTGACTAA</td>
<td>63</td>
</tr>
<tr>
<td>NF1 c.7925del (p.Ser2642fs)</td>
<td>F:TTGGGCATATTCTCTGGGA R:CGCATGTTAGCAAGTTCAC</td>
<td>63</td>
</tr>
<tr>
<td>RB1 c.2212-1G&gt;A</td>
<td>F:CCCTGGCTATTTCTCTCAATC R:AGGATACTTTTGACCTACC</td>
<td>63</td>
</tr>
<tr>
<td>KIF1B c.482delA (p.Lys110fs)</td>
<td>F:CCTAGAGTTACGGCATTTCA R:TACCATACCTGGCTTTGTTC</td>
<td>63</td>
</tr>
<tr>
<td>BRAF c.1318delA (p.Thr440fs)</td>
<td>F:CACAATGTCACACAGTACATAC R:TTCTTAAGGGGTACCTTCTGTA</td>
<td>63</td>
</tr>
<tr>
<td>ERBB2 c.2284delA (p.Lys762fs)</td>
<td>F:TAGGGTGTGAGAGTGTTC R:GAGGATCATACCTTCACCAACC</td>
<td>63</td>
</tr>
<tr>
<td>SDHB c.422T&gt;C (p.Arg91Gly)</td>
<td>F:TAGTAAAGTGTGAGAGTGTTC R:AGCCTCTTGGAGACCACA</td>
<td>63</td>
</tr>
</tbody>
</table>
Table 5.2: The Ion AmpliSeq™ Cancer Panel targets 68 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPL</td>
<td>NRAS</td>
<td>ATP1A1*</td>
</tr>
<tr>
<td>SDHC*</td>
<td>EGLN1*</td>
<td>FH*</td>
</tr>
<tr>
<td>ALK</td>
<td>EPAS1*</td>
<td>TMEM127*</td>
</tr>
<tr>
<td>IDH1</td>
<td>ERBB4</td>
<td>VHL</td>
</tr>
<tr>
<td>MLH1</td>
<td>CTNNB1</td>
<td>CACNA1D*</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>FGFR3</td>
<td>PDGFRA</td>
</tr>
<tr>
<td>KIT</td>
<td>KDR</td>
<td>FBXW7</td>
</tr>
<tr>
<td>SDHA*</td>
<td>APC</td>
<td>CSF1R</td>
</tr>
<tr>
<td>NPM1</td>
<td>EGFR</td>
<td>MET</td>
</tr>
<tr>
<td>SMO</td>
<td>BRAF</td>
<td>EZH2</td>
</tr>
<tr>
<td>FGFR1</td>
<td>JAK2</td>
<td>CDKN2A</td>
</tr>
<tr>
<td>GNAQ</td>
<td>ABL1</td>
<td>NOTCH1</td>
</tr>
<tr>
<td>RET</td>
<td>PTEN</td>
<td>FGFR2</td>
</tr>
<tr>
<td>HRAS</td>
<td>SDHAF2*</td>
<td>MEN1*</td>
</tr>
<tr>
<td>ATM</td>
<td>SDHD*</td>
<td>KCNJ5*</td>
</tr>
<tr>
<td>KRAS</td>
<td>PTPN11</td>
<td>HNF1A</td>
</tr>
<tr>
<td>FLT3</td>
<td>RB1</td>
<td>MAX*</td>
</tr>
<tr>
<td>AKT1</td>
<td>IDH2</td>
<td>CDH1</td>
</tr>
<tr>
<td>TP53</td>
<td>NF1*</td>
<td>ERBB2</td>
</tr>
<tr>
<td>SMAD4</td>
<td>STK11</td>
<td>GNA11</td>
</tr>
<tr>
<td>PRKACA*</td>
<td>JAK3</td>
<td>SMARCB1</td>
</tr>
<tr>
<td>SRC</td>
<td>GNAS</td>
<td>SDHB*</td>
</tr>
<tr>
<td>ATP2B3*</td>
<td>KIF1B*</td>
<td></td>
</tr>
</tbody>
</table>

* = Additional custom hot spot gene regions added.

5.37 Sanger sequencing

DNA extracted from FFPE and FF tumour samples was used for Sanger sequencing using a standard protocol outlined in chapter 2. Sanger sequencing was carried out by research staff in the Department of Academic Medical Genetics and results were analysed using Mutation Surveyor (SoftGenetics), against an appropriate gene specific reference file (GenBank file) downloaded from NCBI.
5.38: Measuring Phenylethanolamine N-methyltransferase (PNMT) gene expression using real-time polymerase chain reaction (RT-PCR)

RNA was isolated from 3 FFPE tumour samples and identified adjacent normal tissue using the RNeasy FFPE RNA Isolation Kit (Qiagen, UK), and cDNA synthesis was performed as outlined in chapter 2. A commercially available PNMT taqman assay was used (Thermofischer, catalogue number 4331182). GAPDH was used as an endogenous control gene. All reactions were performed in triplicates. The amplification protocol was as follows: 95°C for 10 min; 40 cycles of 95°C for 10 s and 60°C for 60 s; and one cycle for melting curve analysis. The relative expression of PNMT compared with that of the reference gene was calculated using ΔCt (cycle threshold) and compared to the PNMT expression of the adjacent normal tissue.

5.39 Statistical analysis
Statistical tests were performed using the MEDCALC medical statistics software (https://www.medcalc.org/). Summary statistics include mean and standard deviation for continuous variables and frequency and percentage for categorical variables.
5.4 Results

5.41: Cohort demographics and clinical features of discovery cohort:

A total of 32 tumour samples (31 primary, 1 metastatic tumour) from 32 patients (20 males, 12 females) were included in the discovery cohort. The mean age was 51 years (SD 19.6, range 15-83 years). The mean tumour size was 5.8cm (SD 3.25, range 1-15cm), 22/32 (68%) tumours were located in the adrenal (PC) and 10 (32%) were extra adrenal abdominal PGL. Seven patients (21.8%) were identified with malignant tumours, five patients had metastases to local regional lymph nodes and two patients had distant metastases (Table 5.3). Clinical genetic testing for germline variants in a panel of ten genes (SDHA, SDHB, SDHC, SDHD, FH, VHL, TMEM127, MAX, RET, SDHAF2) had been performed on 29/32 (91%) individuals prior to this study and identified 12/29 (31%) patients with a genetic predisposition to PPGL (Table 5.3). Finally, a further 38 PPGL samples were sequenced as a validation cohort, resulting in a total of 70 PPGL tumour samples.

5.42: Technical assessment of next generation sequencing assay

The mean read depth was calculated across all samples as 2,437 reads (SD 1057.8) and the mean percentage of reads on target across all samples was 77%. A higher frequency of C>T variants consistent with DNA damage from formalin fixation was noted in the FFPE samples compared to the fresh frozen samples (P=0.0001), however this mutational signature was not significant at a higher allele frequency of >5% (p=0.4).
Table 5.3 Cohort demographics and clinical features of discovery cohort

<table>
<thead>
<tr>
<th>Case #</th>
<th>Sex</th>
<th>Age</th>
<th>Tumour location</th>
<th>Tumour size (cm)</th>
<th>Malignant</th>
<th>Pass score</th>
<th>Secretory pattern</th>
<th>Germline pathogenic variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>23</td>
<td>Adrenal</td>
<td>3</td>
<td>No</td>
<td>0</td>
<td>Noradrenergic</td>
<td>VHL c.499C&gt;G p.Arg167Gly</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>31</td>
<td>Adrenal</td>
<td>4.4</td>
<td>No</td>
<td>4</td>
<td>Mixed</td>
<td>No mutation</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>19</td>
<td>Abd PGL</td>
<td>6</td>
<td>No</td>
<td>4</td>
<td>Noradrenergic</td>
<td>SDHB c.380G&gt;T p.Ille127Ser</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>77</td>
<td>Adrenal</td>
<td>6</td>
<td>No</td>
<td>8</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>70</td>
<td>Adrenal</td>
<td>6</td>
<td>No</td>
<td>2</td>
<td>Adrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>27</td>
<td>Abd PGL</td>
<td>7</td>
<td>No</td>
<td>3</td>
<td>Non secretory</td>
<td>SDHB c.302G&gt;A p.Cys101Tyr</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>35</td>
<td>Adrenal</td>
<td>12</td>
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<td>10</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>48</td>
<td>Adrenal</td>
<td>2</td>
<td>No</td>
<td>1</td>
<td>Adrenergic</td>
<td>RET c.1900T&gt;A p.Cys634Ser</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>15</td>
<td>Abd PGL</td>
<td>7</td>
<td>No</td>
<td>1</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>22</td>
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<td>5</td>
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<td>No mutation</td>
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<tr>
<td>11</td>
<td>M</td>
<td>62</td>
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<td>8</td>
<td>No</td>
<td>7</td>
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<td>No mutation</td>
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<tr>
<td>12</td>
<td>F</td>
<td>37</td>
<td>Adrenal</td>
<td>6.5</td>
<td>No</td>
<td>5</td>
<td>Adrenergic</td>
<td>RET c.1900T&gt;A p.Cys634Ser</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
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<td>Adrenal</td>
<td>2</td>
<td>No</td>
<td>2</td>
<td>Noradrenergic</td>
<td>NF1 c.1318C&gt;T p.Arg440Ter</td>
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<tr>
<td>14</td>
<td>F</td>
<td>59</td>
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<tr>
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<td>F</td>
<td>78</td>
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<td>3</td>
<td>Noradrenergic</td>
<td>SDHC c.43C&gt;T p.Arg15*</td>
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<tr>
<td>16</td>
<td>M</td>
<td>68</td>
<td>Abd PGL</td>
<td>5</td>
<td>Yes</td>
<td>NA</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
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<td>Adrenal</td>
<td>12</td>
<td>No</td>
<td>14</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>59</td>
<td>*Rib metastases</td>
<td>1</td>
<td>Yes</td>
<td>NA</td>
<td>Noradrenergic</td>
<td>SDHA c.91C&gt;T p.Arg31Ter</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>46</td>
<td>Adrenal</td>
<td>15</td>
<td>Yes</td>
<td>15</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>66</td>
<td>Adrenal</td>
<td>5</td>
<td>No</td>
<td>1</td>
<td>Noradrenergic</td>
<td>No mutation</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>68</td>
<td>Abd PGL</td>
<td>11</td>
<td>No</td>
<td>NA</td>
<td>Non secretory</td>
<td>No mutation</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>83</td>
<td>Adrenal</td>
<td>4</td>
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<td>4</td>
<td>Noradrenergic</td>
<td>Not tested</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>31</td>
<td>Adrenal</td>
<td>4</td>
<td>No</td>
<td>3</td>
<td>Non secretory</td>
<td>No mutation</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>44</td>
<td>Abd PGL</td>
<td>1.5</td>
<td>Yes</td>
<td>NA</td>
<td>Noradrenergic</td>
<td>SDHB c.268C&gt;G p.Arg90Gly</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>69</td>
<td>Adrenal</td>
<td>8</td>
<td>No</td>
<td>9</td>
<td>Noradrenergic</td>
<td>SDHA c.91C&gt;T p.Arg31Ter</td>
</tr>
</tbody>
</table>
5.43: Tumour sequencing of inherited PPGL cases

Twelve cases of hereditary PPGL (2 malignant, 10 benign) were included in the discovery cohort and analysed for evidence of loss of heterozygosity in the tumour and or the presence of additional somatic driver variants. All but one tumour had evidence of loss of heterozygosity (LOH). The tumour without LOH at the germline locus (case #18), was a secondary metastases in a patient with a germline SDHA variant (c.91C>T p.Arg31Ter) (Table 5.4). Although no LOH was identified in this tumour, an additional truncating somatic variant in SDHA (c.1308G>T (p.Glu424X) (VAF 27%) and a further somatic missense variant in SDHB (c.344G>A (p.Arg115Gln) (VAF of 11%) was identified in the tumour. Notably, a third potential driver variant in the proto-oncogene KRAS (c.88C>T p.Asp30Asn) was also discovered in this tumour sample. No additional somatic driver variants were identified in the remaining 11 hereditary primary PPGL tumour samples.
Table 5.4: Tumour sequencing of inherited PPGL cases

<table>
<thead>
<tr>
<th>Inherited pathogenic variant</th>
<th>Case number</th>
<th>Loss of heterozygosity</th>
<th>Identified somatic driver variants (Variant allele frequency %)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>SDHB</em></td>
<td>#3</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>#6</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>#14</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>#24</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><em>SDHA</em></td>
<td>#18</td>
<td>No</td>
<td><em>SDHA</em> c. c.1270G&gt;T (p.Glu424X) (27%) <em>SDHB</em> c.344G&gt;A (p.Arg115Leu) (11%) <em>KRAS</em> c.88C&gt;T (p.Asp30Asn) (13%)</td>
</tr>
<tr>
<td><em>SDHA</em></td>
<td>#25</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><em>SDHC</em></td>
<td>#15</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><em>VHL</em></td>
<td>#1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><em>NF1</em></td>
<td>#13</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><em>RET</em></td>
<td>#8</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>#12</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><em>MAX</em></td>
<td>#28</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

5.44: Somatic mutation landscape of discovery and validation tumour cohorts

The mean variant count was 1,761 before filtering and 37.6 after filtering in the 70 PPGL samples. Post variant filtering, the number of somatic variants with a VAF of ≥1% was calculated for each tumour sample and defined for this study as the ‘somatic mutation burden’ (SMB). There was no significant difference in SMB noted for; i) adrenal phaeochromocytoma compared to extra adrenal paraganglioma tumours (p=0.8), ii) benign versus malignant tumours (P=0.4), or iii) hereditary versus sporadic tumours. A higher SMB was identified in the FFPE samples compared to fresh frozen (p=0.01) (Figure 5.3) but this finding was not significant at a VAF >5% (P=0.067), suggesting that many of the variants detected at a VAF between 1-5% were potential sequence artefacts.
**i) Discovery tumour cohort**

For each tumour sample in the discovery set, the somatic status of an identified variant was confirmed by the absence of that variant in the matched constitutional DNA. A potential pathogenic driver variant was identified and validated by Sanger sequencing in 10/32 (31%) cases in the discovery cohort and aside from one case (case #18), the remaining 12 tumours were cases of sporadic PPGL (10 phaeochromocytoma, 2 abdominal paraganglioma).

Three sporadic PPGL (9.4%) harboured a somatic novel \textit{NF1} frameshift variant. An activating missense variant in the \textit{HRAS} hotspot region of codon 61 was identified in two tumours (6.2%) and two known pathogenic missense variants in \textit{VHL} were identified in a further two tumours (6.2%) (Table 5.5). A somatic missense variant in the hotspot region of codon 132 in \textit{IDH1} was discovered in a single sporadic paraganglioma and a pathogenic splice site variant in \textit{SDHB} was identified in the second sporadic paraganglioma. Finally, novel frameshift variants in \textit{KIF1B, FBXW7} and \textit{SMO} were identified in the remaining three tumours (Table 5.5). Somatic variants in \textit{SMO} and \textit{FBXW7} have not previously been implicated in PPGL.

Sanger sequencing did not confirm the variants in \textit{FBXW7, SMO} or \textit{KIF1B}. However, there was sufficient DNA from sample #19 with the FBXW7 variant and sample #32 with the KIF1B variant to proceed with targeted NGS analysis using a capture panel of 350 genes. Repeat sequencing using this method did not identify the \textit{KIF1B} variant but the \textit{FBXW7} variant in case #19 was detected again using this validatory sequencing method (Figure 5.3).
Table 5.5: Somatic mutations identified by next generation sequencing in discovery tumour set

NP= not performed due to VAF (<15%) below diagnostic threshold for Sanger sequencing

<table>
<thead>
<tr>
<th>Inherited pathogenic variant</th>
<th>Case number</th>
<th>Gene</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Variant allele frequency %</th>
<th>Validated by Sanger sequencing or target sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>#5</td>
<td>HRAS</td>
<td>c.182A&gt;C</td>
<td>(p.Gln61Pro)</td>
<td>29%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#7</td>
<td>SMO</td>
<td>c.1576delT</td>
<td>(p.Phe526fs)</td>
<td>15%</td>
<td>No</td>
</tr>
<tr>
<td>No</td>
<td>#9</td>
<td>SDHB</td>
<td>c.423+1G&gt;A</td>
<td></td>
<td>15%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#10</td>
<td>VHL</td>
<td>c.482G&gt;A</td>
<td>(p.Arg161Gln)</td>
<td>35%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#11</td>
<td>NF1</td>
<td>c.2927_2933delCTGAAGG</td>
<td>(p.Thr976fs)</td>
<td>36%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#16</td>
<td>IDH1</td>
<td>c.394C&gt;T</td>
<td>(p.Arg132Cys)</td>
<td>40%</td>
<td>Yes</td>
</tr>
<tr>
<td>SDHA</td>
<td>#18</td>
<td>SDHA</td>
<td>c.1270G&gt;T</td>
<td>(p.Glu424X)</td>
<td>27%</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDHB</td>
<td>c.344G&gt;A</td>
<td>(p.Arg115Leu)</td>
<td>11%</td>
<td>NP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KRAS</td>
<td>c.88G&gt;A</td>
<td>(p.Asp30Asn)</td>
<td>13%</td>
<td>NP</td>
</tr>
<tr>
<td>No</td>
<td>#19</td>
<td>FBXW7</td>
<td></td>
<td>(p.Cys384fs)</td>
<td>80%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#20</td>
<td>VHL</td>
<td>c.245G&gt;T</td>
<td>(p.Arg82Leu)</td>
<td>22%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#27</td>
<td>HRAS</td>
<td>c.182A&gt;C</td>
<td>(p.Gln61Pro)</td>
<td>41%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#29</td>
<td>NF1</td>
<td>c.7925delC</td>
<td>(p.Ser2642fs)</td>
<td>15%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#30</td>
<td>NF1</td>
<td>c.2098delA</td>
<td>(p.Thr700fs)</td>
<td>40%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#32</td>
<td>KIF1B</td>
<td>c.482delA</td>
<td>(p.Lys110fs)</td>
<td>20%</td>
<td>No</td>
</tr>
</tbody>
</table>
**Figure 5.3:** Figure A shows a sagittal section from an 18F-FDG-PET CT showing lung metastases (as illustrated by the white arrows) in case #19 with a metastatic phaeochromocytoma. Figure B shows the somatic *FBXW7* frameshift variant in the primary tumour from case#19 and illustrated on integrated genomic viewer with a variant allele frequency of 80%.

---

**ii) Validation cohort**

Matched constitutional DNA was not available for the tumour samples in the validation cohort. A potential driver somatic variant was identified and confirmed by Sanger sequencing in 2/38 samples (5.2%). Two different missense variants in *NF1* were identified in two sporadic PPGL (5.2%) but these two *NF1* variants were not validated by Sanger sequencing as the variant allele frequency of these two *NF1* variants was below the threshold for detection by Sanger sequencing (<15%). Similarly a variant with a VAF <15% was detected in *PIK3CA* and *APC*, which again could not be validated by Sanger sequencing and a novel frameshift variant in *BRAF* was identified in a single sample, which due to insufficient DNA could not be validated by Sanger sequencing. Variants in two other genes, also identified as harbouring somatic variants in the discovery cohort, were identified in the validation cohort and validated by Sanger sequencing including; i) a
novel frameshift variant in *VHL* (c.413_414delCA, p.Pro138fs) and ii) a missense variant in *SDHB* (c.422T>C, p.Arg91Gly) in two sporadic PPGL (Table 5.6). The confirmation of a splice site variant in *RB1* was classified as indeterminate by Sanger sequencing due to the proximity of a homopolymeric region and the low VAF (15%). Finally a novel frameshift variant in *ERBB2* was not validated by Sanger sequencing or targeted sequencing and was subsequently classified as a presumed sequencing artefact.
Figure 5.4: Figure A shows the frequency of a gene harbouring a validated driver variant in the 70 PPGL cases in this study and figure B shows data adapted from COSMIC (https://cancer.sanger.ac.uk/cosmic), highlighting the top 20 most common genes to harbour a somatic variant in PPGL.

Table 5.6: Somatic variants identified by next generation sequencing in the validation cohort.

NP= not performed due to VAF (<15%) below diagnostic threshold for Sanger sequencing

<table>
<thead>
<tr>
<th>Inherited pathogenic variant</th>
<th>Case number</th>
<th>Gene</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Variant allele frequency %</th>
<th>Validated by Sanger sequencing or targeted sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>#39</td>
<td>NF1</td>
<td>c.2392A&gt;C</td>
<td>p.Lys798Gln</td>
<td>6%</td>
<td>NP</td>
</tr>
<tr>
<td>No</td>
<td>#40</td>
<td>NF1</td>
<td>c.2690G&gt;A</td>
<td>p.Arg897Gln</td>
<td>6%</td>
<td>NP</td>
</tr>
<tr>
<td>No</td>
<td>#40</td>
<td>PIK3CA</td>
<td>c.2177delA</td>
<td>p.Glu726fs</td>
<td>6%</td>
<td>NP</td>
</tr>
<tr>
<td>No</td>
<td>#43</td>
<td>APC</td>
<td>c.4328delA</td>
<td>p.Glu1443fs</td>
<td>6%</td>
<td>NP</td>
</tr>
<tr>
<td>No</td>
<td>#49</td>
<td>VHL</td>
<td>c.413-414delCA</td>
<td>p.Pro138fs</td>
<td>27%</td>
<td>Yes</td>
</tr>
<tr>
<td>SDHA</td>
<td>#56</td>
<td>RB1</td>
<td>c.2212-1G&gt;A</td>
<td></td>
<td>15%</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>SDHD</td>
<td>#58</td>
<td>BRAF</td>
<td>c.1318delA</td>
<td>p.Thr440fs</td>
<td>17%</td>
<td>No (insufficient DNA)</td>
</tr>
<tr>
<td>No</td>
<td>#68</td>
<td>SDHB</td>
<td>c.422T&gt;C</td>
<td>p.Arg91Gly</td>
<td>14%</td>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
<td>#69</td>
<td>ERBB2</td>
<td>c.2284delA</td>
<td>p.Lys762fs</td>
<td>20%</td>
<td>No</td>
</tr>
</tbody>
</table>
5.45: Relationship between clinical phenotype and somatic driver variants in discovery cohort

No significant difference was observed for mean age at presentation (51.5 years v’s 49 years, p=0.04) or tumour size (8cm v’s 5.5cm, p=0.15) in those patients identified with a somatic driver variant compared to those patients without. Chi-squared analysis determined that sporadic tumours were more likely to harbour a somatic driver variant compared to those patients with an identified genetic predisposition (p=0.04) but no difference was noted for malignant PPGL (p=0.6) or tumour location (p=0.5). Similar to previous studies, no somatic driver variants in HRAS were identified in the 12 (38%) cases of hereditary PPGL, reiterating the observation that variants in HRAS and known hereditary PPGL genes are mutually exclusive drivers of tumourigenesis (209). As previously reported, a predominant adrenergic secretory phenotype was noted for those cases with an identified HRAS variant (cases #05 and #27) and a predominant noradrenergic secretory phenotype was noted for the two cases with an identified somatic VHL variant in the discovery cohort (cases #10 and #20) in this study.

It is well established for hereditary PPGL that the pattern of catecholamine secretion correlates with the molecular drivers of tumourigenesis. Germline variants in the ‘cluster 1’ genes are associated with a predominant noradrenergic secretory pattern, due to downregulation of the enzyme PNMT (phenylethanolamine-N-methyltransferase) responsible for the conversion of norepinephrine to epinephrine (240). In this study, a predominant noradrenergic secretory phenotype was noted for the two cases with an identified somatic VHL variant in the discovery cohort (cases #10 and #20). In order to confirm if PNMT expression in these tumours was affected by the presence of a somatic VHL variant as has been described for germline VHL variants(240), PNMT expression was measured in the tumour and adjacent normal tissue for case #10 (case #20 was not tested due to insufficient
tumour cDNA) and compared to PNMT expression levels in the tumour from case #1 with a pathogenic germline VHL variant (c.499C>G, p.Arg167Gly) and case #12 with a pathogenic germline RET variant (c.1900T>A, p.Cys634Ser) (Figure 5.7D). PNMT expression was significantly lower in the tumour tissue compared to the adjacent normal tissue in case #10 (p=0.002) and was comparable to the expression of PNMT in the tumour of case #1 with a germline VHL variant (p=0.12) supporting the association of the pathogenic somatic VHL variant in case #10 with the noradrenergic secretory phenotype (Figure 5.5).

**Figure 5.5:** Figure A demonstrates the large right sided phaeochromocytoma from case#10 highlighted by the white arrow. Figure B is the chromatogram from Sanger sequencing confirming the somatic pathogenic missense VHL variant (c.482G>A, p.Arg161Gln) as highlighted by the black arrow. Figure C shows the predominant noradrenergic secretory phenotype in case #10. Figure D illustrates the PNMT expression in tumour and adjacent normal tissue from case #10 compared to case #1 with a germline VHL variant and case #16 with a germline RET variant.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma normetadrenaline</td>
<td>34,000 pmol/L</td>
<td>&lt;2000 pmol/L</td>
</tr>
<tr>
<td>Plasma metadrenaline</td>
<td>&lt;180 pmol/L</td>
<td>&lt;1500 pmol/L</td>
</tr>
</tbody>
</table>
5.46: Molecular pathways affected by identified pathogenic somatic variants

The identification of somatic driver variants can aid in the prediction of affected molecular pathways and cellular functions, with the purpose of identifying molecular mechanisms of tumourigenesis, which can be targeted for therapeutic intervention and or used as predictive biomarkers of biological behaviour. It is intriguing that variants in the ‘cluster 1 genes’ are more commonly implicated in hereditary PPGL, whereas somatic variants in sporadic PPGL occur with a greater frequency in the ‘cluster 2’ genes (Figure 5.6).

I analysed the somatic driver variants identified in this study (including both discovery and validation cohorts) using Gene Ontology (http://geneontology.org/) and Gene Mania (https://genemania.org/) to identify if the mutated genes identified in this study, converged onto interacting gene pathways. Indeed, using the Gene Ontology Enrichment analysis tool HRAS, NF1 and VHL were mapped to functions including regulation of intracellular signal transduction (FDR = 0.0473) and regulation of apoptotic process (FDR = 0.0328). In particular, HRAS and NF1 are components of the RAS signalling pathways (RAS/RAF/MAPK and PI3K/AKT/mTOR) at different levels. These three genes converged onto pathways involved in GTPase mediated signal transduction (FDR = 0.0328). (Table 5.7). Notably in this study, 7/12 (58.3%) of the identified somatic variants occurred in ‘cluster 1’ genes (3 variants in VHL, 2 in SDHB, 1 SDHA and 1 in IDH1).
**Figure 5.6:** This figure illustrates the estimated contribution of somatic (data adapted from COSMIC) and germline variants (adapted from (241)) in the most common PPGL susceptibility genes.

**Table 5.7:** Molecular pathways predicted to be affected by variants in the genes NF1, HRAS and VHL by Gene Mania (https://genemania.org/)

<table>
<thead>
<tr>
<th>Molecular pathway</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive regulation of peptidyl-serine phosphorylation</td>
<td>0.016</td>
</tr>
<tr>
<td>regulation of ERK1 and ERK2 cascade</td>
<td>0.005</td>
</tr>
<tr>
<td>phosphatidylinositol 3-kinase signalling</td>
<td>0.00002</td>
</tr>
<tr>
<td>protein kinase activator activity</td>
<td>0.005</td>
</tr>
<tr>
<td>protein serine/threonine kinase activity</td>
<td>0.00006</td>
</tr>
<tr>
<td>regulation of Ras GTPase activity</td>
<td>.00007</td>
</tr>
<tr>
<td>regulation of cell adhesion</td>
<td>0.03</td>
</tr>
<tr>
<td>peptidyl-serine phosphorylation</td>
<td>0.006</td>
</tr>
</tbody>
</table>
5.5 Discussion:

5.51: Translational utility of somatic profiling in PPGL

A candidate somatic driver variant was identified and validated in 19% (13/70) of cases in this study and with the exception of a single case (#18), somatic driver variants occurred exclusively in sporadic tumours and this suggests (similar to findings reported by other larger studies (210), that somatic sequencing may be best utilised in those patients with sporadic PPGL. The frequency of somatic variants detected in this study is lower than that reported in the literature (210) and this may be explained by the exclusion of variants in this study that were not or could not be validated by Sanger sequencing or an alternative orthogonal validation method.

It is noteworthy that the genes most commonly affected by somatic variants in PPGL fall predominately into the ‘cluster 2’ category of genes and this is in stark contrast to those genes most commonly implicated in hereditary PPGL, which fall into the ‘cluster 1 pathway’ (Figure 5.6). However, the findings in this study are in contrast to previous studies as 58% of detected variants in this study were in ‘cluster 1’ genes.

Similar to the COSMIC somatic variant frequency data for PPGL, variants in NF1, VHL and HRAS were among the most common identified in this study. Variants in both NF1 and HRAS affect the RAS/RAF/ERK and therefore the identification of driver variants in these genes raises the possibility of therapeutic targeting with agents such as ERK1/2 inhibitors for patients with malignant PPGL (242). However, no case of metastatic PPGL with a somatic driver variant in HRAS or NF1 was identified in this study and cases in the literature are rare (209)(212)(213)(215). Therefore, this suggests that the identification of somatic variants in NF1 and HRAS may be more useful in predicting a more benign tumour and stratifying long
term surveillance rather than informing therapeutic options, but larger prospective studies would be required to confirm this hypothesis. The *ATRX* gene was not included on the 68 gene panel employed in this study, however there is a suggestion that pathogenic somatic variants in *ATRX* are associated with malignant PPGL (222). In this study by Fishbein and colleagues, five out of six tumours with an identified pathogenic somatic *ATRX* variant were clinically aggressive (222).and therefore diagnosis of a somatic *ATRX* variant may provide valuable additional prognostic information for sporadic PPGL. Finally, identifying a driver somatic variant can aid genetic counselling by helping to relieve anxiety regarding a potential hereditary predisposition.

Finally, this study identified a novel somatic frameshift variant in *FBXW7* in a patient with a metastatic phaeochromocytoma. *FBXW7* encodes a member of the F-box protein family and is also a subunit component of the ubiquitin protein ligase complex, which plays a role in phosphorylation-dependent ubiquitination (243). Although somatic variants in *FBXW7* have been implicated in a number of cancers including ovarian, breast and colorectal (244), no variant in *FBXW7* has been identified in 235 phaeochromocytoma samples (230 benign and 5 malignant) reported on COSMIC. Interestingly, loss of function of the FBXW7 gene has been implicated in renal cell carcinoma in the literature (245) and as demonstrated in chapter 4, there is a significant overlap in the genetic architecture of PPGL and RCC. Studies have demonstrated that loss of function of *FBXW7* results in an increase of mTOR, p-mTOR and S6-kinase (p-SK6) (246), and this raises the possibility that inactivation of *FBXW7* may be used as a biomarker to predict clinical response to mTOR inhibitors (247). Further analysis is required to determine the downstream signalling effects of the *FBXW7* variant identified in this case.
5.52: The role of targeted next generation sequencing assays in clinical diagnostics and associated pitfalls

The clinical application of next-generation sequencing (NGS) assays for detection of somatic variants offers the potential to improve patient diagnosis, prognosis, and treatment options. The benefit of targeted gene panels for somatic sequencing such as the panel employed in this study include; i) cost effectiveness, ii) lower DNA concentration requirements compared to whole exome or whole genome sequencing strategies and iii) higher mean coverage, which enables variants with a lower VAF to be detected.

However, profiling of tumours by NGS remains challenging. Firstly the quality and quantity of DNA can vary depending on the tumour samples received (FF versus FFPE)(248). In this study, a higher frequency of C>T variants was noted in the FFPE samples compared to the FF samples (P=0.0001) but this was not significant at a higher allele frequency of >5% (p=0.4). Sequencing artefacts can be attributed to various factors including PCR amplification bias and issues with calling algorithms. Furthermore artefactual insertion and deletion (INDEL) variants are also a common challenge associated with NGS technology in homo-polymeric sequencing regions (248). In this study three novel frameshift variants in *FBXW7*, *KIF1B* and *ERBB2* were detected by NGS but were not validated by Sanger sequencing. Two of these variants were subsequently classified as presumed sequence artefacts but it is notable that all three variants had a VAF >20%, had a mean coverage of >850 reads but two of the variants (*KIF1B* and *ERBB2*) were in homo-polymeric regions, assessed using the RepeatMasker tool on USCS Genome browser (https://genome.ucsc.edu). These findings highlight the challenge of differentiating potential novel somatic variants including potential loss of function INDEL variants detected by NGS from sequence artefacts. The validation of detected germline variants is traditionally performed by Sanger sequencing. However, Sanger sequencing of tumour DNA also poses technical challenges related to DNA fragmentation and the
sensitivity of Sanger sequencing to detect variants at a low VAF (<15%) as noted in this study. In this study, 6/24 (25%) variants detected had a VAF <15% and could not be validated by Sanger sequencing and a further indeterminate result was demonstrated for a splice site variant in RB1. As Sanger sequencing is not the ideal orthogonal validation method for validating detected somatic variants by NGS, one option would be to consider alternative methods of target enrichment such as hybrid capture-based targeted sequencing for variant validation or as the primary sequencing technique for somatic tumour profiling. Hybrid capture-based sequencing has demonstrated superior sensitivity compared to amplicon-based sequencing and maintains more uniform coverage with fewer sequencing artefacts related to library construction of degraded DNA such as FFPE DNA (248). However, one drawback of capture based sequencing methods is the requirement for higher DNA concentrations compared to amplicon based methods. In this study a capture based sequencing method validated a FBXW7 variant in case #19 and confirmed that the two additional novel frameshift variants in ERBB2 and KIF1B were sequence artefacts.

Therefore, if a targeted gene sequencing panel is to be considered for clinical utility, careful consideration of issues including: i) the role of replicate Sanger sequencing versus alternative sequencing methods for variant validation and ii) the value of expanding the 68 gene panel used in this study to include genes recently implicated in PPGL such as MDH2 (67), SCL25A11 (249) and ATRX (222), is required.
5.6 Conclusion

This study has consolidated the role of *NF1*, *HRAS* and *VHL* somatic driver variants in PPGL and confirms the apparent mutual exclusivity of somatic and germline driver variants in PPGL. A pathogenic somatic variant in *FBXW7* in a metastatic phaeochromocytoma, was identified for the first time in this study and raises the possibility of selective targeted treatment options for this case.

Finally, this study has also highlighted technical issues related to tumour sequencing and variant validation using a targeted next generation sequencing approach. Therefore despite the accepted clinical applications of PPGL tumour sequencing, further prospective studies are required to determine the optimum sequencing strategy and post-analytic interpretation methodologies, before PPGL somatic profiling can be considered for routine clinical use.
Chapter 6
Investigating the clinical applications of ex-
vivo metabolomics profiling
Chapter 6.0: Investigating the clinical applications of *ex-vivo* metabolomics profiling

6.1 Introduction

6.11 Application of metabolomics analysis in clinical practice

6.12 Metabolomics analysis in PPGL
6.13 Scientific techniques employed in metabolite profiling
6.14 High resolution magic angle spinning (HRMAS) NMR spectroscopy

6.2 Aims

6.3 Methods

6.31 Clinical sample collection
6.32 Germline genetic analysis
6.33 Tumour sequencing
6.34 HRMAS NMR spectroscopy and data analysis
6.35 Liquid chromatography mass spectroscopy
6.36 Statistical analysis
6.37 SDHB Immunohistochemistry

6.4 Results

6.41 Genotype and clinical phenotype of cohort of patients with GIST and PPGL
6.42 Succinate detection ex vivo
6.43 Untargeted metabolic profiling of SDH deficient tumours
6.44 Unmasking of a somatic IDH1 pathogenic variant by untargeted metabolomics profiling
6.45 Measurement of 2-Hydroxyglutarate in *IDH-1* mutant glioma samples
6.46 Comparing the metabolomics profiles of SDH deficient and *IDH1* mutant tumours

6.5 Discussion

6.51 Clinical utility of ex-vivo metabolomics analysis using HRMAS in GIST and PPGL
6.52 Metabolic reprogramming in *SDH* mutated tumours
6.53 Limitations of HRMAS NMR spectroscopy

6.6 Conclusion

6.1 Introduction
**6.11 Application of metabolomics analysis in clinical practice**

Metabolomics refers to the study of metabolites within a biological system and is a relatively new ‘omics’ science. Investigating the metabolome of cancer cells provides an insight into changes in phenotype allowing for dynamic interpretation of tumour biology compared to other ‘omics’ technologies. The study of a tumour metabolome has the potential to have translational application as an early disease biomarker, and/or predictor of pharmacotherapy response and resistance. Metabolomic profiling like genetic sequencing can be targeted or untargeted, with untargeted approaches favoured for novel biomarker discovery in human disease. In 2004, the Human Metabolome Project (HMP)(250), the equivalent of the Human Genome Project for metabolomics, was established and recently in 2013, the third and updated version of the Human Metabolome Database (HMDB) was released and provides an inventory of over 40,000 identified human metabolites (251).

Metabolomic profiling has been applied to a number of common cancers including prostate cancer and breast cancer and prostate cancer in particular is often used as a paradigm for the potential clinical applications of this ‘omics’ science. A feature of normal prostate cells is the production of the metabolite citrate and metabolomic profiling has demonstrated that the concentration of citrate decreases in both prostate cells and seminal fluid when prostate cells undergo neoplastic transformation (252). This metabolomic signature of prostate cancer has been replicated in *ex-vivo* and *in-vivo* (253) analyses and has demonstrated efficacy as a biomarker, showing good correlation with Gleason scores in the diagnosis of prostate cancer (252)(253).

Furthermore, metabolomics profiling can inform the metabolic reprogramming that is prioritised by cancer cells in order to achieve a growth and proliferation advantage and in turn can provide information on aberrant oncogenes and tumour suppressor genes, which orchestrate this re-programming (254). The aim is that this data can ultimately be applied to
the development of precision metabolomic diagnostics to inform management and targeted therapeutics. The translational applications of metabolomic profiling has been elegantly demonstrated in myeloid leukaemia and gliomas caused by activating somatic variants in the gene isocitrate dehydrogenase type 1 (IDH1). Pathogenic variants in IDH1 result in the accumulation of the metabolite 2-hydroxyglutarate (2HG) and measurement of this metabolite both in vivo and ex vivo in glioma tumours, has demonstrated efficacy as a biomarker to aid the development of tailored management and surveillance protocols, as IDH1 mutations confer a better prognosis compared to IDH1 wild type tumours (255) (256) (257).

6.12 Metabolomics analysis in PPGL

Global metabolite profiling has the potential to guide genetic testing in hereditary disease such as PPGL and in particular tumours caused by loss of function of the SDH enzyme complex. SDH deficient tumours result in a truncated citric acid cycle and therefore are the epitome of Warburg’s original theory of altered cell metabolism in cancer (258). The accumulation of the ‘oncometabolite’ succinate as a result of SDH deficiency is believed to be the main driver in tumourigenesis and studies have demonstrated that succinate concentrations are elevated 25-fold in SDHx mutated tumours compared to non-SDHx mutated tumours (259) and that detection of a high succinate to fumarate ratio in tumour tissue can be used as a predictive test to guide molecular genetic testing (259). Furthermore a unique metabolomic fingerprint has emerged for SDHx mutated PPGL, namely an elevated succinate, glutamine and myoinositol concentration and a lower aspartate, glutamate, fumarate, citrate and isocitrate level (259)(260).
In theory metabolomic profiling of PPGL, might be used to guide genetic testing and also provide an additional functional tool for the assessment of variants of uncertain significance in *SDHx* genes. Metabolomic assessment would be of particular value in the assessment of novel *SDHx* missense variants that might disrupt enzyme function without affecting expression of the SDHB protein, and produce normal results for SDHB immunohistochemistry. Metabolomics may also reveal SDH dysfunction in tumors with a somatic *SDH* variant or those without a detectable sequence alteration such as those tumors caused by epigenetic inactivation of the *SDHC* gene (260). Finally, identifying a unique metabolomic fingerprint in genetically driven PPGL may reveal metabolic vulnerability, which could ultimately be targeted for therapeutic discovery.

6.13 Scientific techniques employed in metabolomic profiling of tumours

The two main analytical technologies employed in the measurement of tissue metabolites are nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS).

MS is regarded as the most sensitive analytical technique and is coupled with gas chromatography (GC) or liquid chromatography (LC) to segregate metabolites. LC-MS has demonstrated high sensitivity and is capable of detecting a large size range of metabolites at room temperature (261). One major drawback for solid tissue analysis using LC-MS, is the requirement for the tissue to be prepared into a solution prior to analysis. This preparation process has the potential to modify the integrity of metabolites, therefore adding further variability to a complex technique.

$^1$H NMR spectroscopy is modelled on the ability to measure electromagnetic radiation emitted by the nuclei of certain isotopes when placed in a high magnetic field. This electrical energy or charge is created by the spin of a nucleus and defined as a ‘spinning state’ (262).
6.14 High resolution magic angle spinning (HRMAS) NMR spectroscopy

High-resolution magic angle spinning (HRMAS) $^1$H NMR spectroscopy technology is particularly useful for the assessment of small volume, intact tissue samples as it does not require chemical extraction procedures or sample manipulation (262). HRMAS inherited its name from the angle that a sample is placed at in relation to the magnetic field, in order for the ‘spin state’ to produce a good quality spectra from a solid tissue sample. Additional advantages of this analytical technique include, rapid preparation (3-4 minutes) and spectral acquisition (~15 minutes) time without affecting spectra quality or resolution (262). HRMAS $^1$H NMR spectroscopy has demonstrated efficacy as a tool for metabolomic profiling in prostate cancer (263), glioma (255) and more recently in PPGL (260).

Figure 6.10: A flow chart of the translational utility of ‘omics’ science
6.2 Aims:

1. To determine if HRMAS can be applied to detect succinate accumulation *ex-vivo* in PPGL and GIST tumour samples and to identify the relevant clinical utility of this technique.

2. To perform untargeted metabolomic profiling of GIST and PPGL tumours in order to investigate if there are unique metabolomic fingerprints associated with specific genotypes.

3. To determine if the metabolomic fingerprint of *IDH1* mutant glioma tumours overlaps with that of SDH deficient GIST and PPGL.

6.3 Methods:

6.31 Clinical sample collection

A sample of 15-20mg of fresh frozen tumour was collected on 75 tumour samples (35 glioma, 8 GIST and 32 PPGL samples) from 75 individuals after informed consent. The tumour samples were retrieved from the Cambridge University NHS Foundation Trust tissue bank and the GIST Support UK National tissue bank. All tissue samples were retrieved after inspection by a pathologist and stored at -80°C in the respective tissue bank within four hours of surgical procedure. None of the patients received pre-treatment with an anti-proliferative therapy prior to surgery and tumour removal. After retrieval from the tissue bank, samples were stored at -80°C until the time of NMR analysis.
6.32 Germline genetic analysis

Data on the germline genetic testing results was available for 38/40 (95%) cases with GIST or PPGL, 31/38 (82%) cases had germline genetic sequencing performed (see methods section, chapter 2). The remaining (7/38) cases had germline genetic analysis performed at another UK institution or had no genetic analysis performed and results when available, were provided to us on referral and after written patient consent.

6.33 Tumour sequencing

See methods section, chapter 2.

6.34 HRMAS NMR spectroscopy:

This analysis was performed by Dr Madhu Basetti in the Cambridge CRUK institute.

HRMAS $^1$H NMR data acquisition was performed on a Bruker Avance 600 MHz NMR instrument with a 4 mm HRMAS probe (Bruker, Germany). All the spectra were obtained using the TOPSPIN 2.16 software (Bruker, Germany) and at a spin rate of 3000 Hz and a sample temperature of 4°C. A water-suppressed pulse sequence with a repetition time of 8 s, 128 transients and 64K time domain points was used to acquire the metabolite spectrum. The corresponding water spectrum was acquired with 8 s repetition time, 8 transients and 64K time domain points. A water-suppressed CPMG pulse sequence was used with a T2 filter (50/100/200 ms) to acquire metabolite spectra with suppression of the broad lipid and macromolecule signals; acquisition parameters: 8 s repetition time, 128 transients, 64K time domain points, total analysis time per sample was approximately 90 min. Absolute metabolite concentrations were estimated by fitting the metabolite signals in the water-suppressed
HRMAS $^1$H NMR spectrum in LCModel and using tissue water signal as internal standard for absolute concentration (15). For each metabolite, LCModel reports both peak area and estimated uncertainty in fitting of the peak (percent standard deviation [SD]). Succinate was interpreted as detectable if the percent SD was < 5% and negative if it was > 5%.

Two-dimensional (2D) homonuclear ($^1$H-$^1$H) NMR experiments were also performed by Dr Madhu Basetti, to validate the detection of specific metabolites on 1D spectra acquisition. Because the duration of these experiments is long and tissue degradation occurs during NMR acquisition, not all representative samples were analyzed by 2D experiments. Metabolites were assigned using standard metabolite chemical shift tables available in the literature (251). Certain metabolites were detected in some, but not all, samples, therefore metabolites that were detected in <50% of replicates were excluded but those metabolites detected in >50% of tumour samples but not detected in all samples, were taken to be below the threshold for detection in those samples and assigned the lowest detected concentration level. Principal component analysis was performed to determine the quality of the data and to optimize the separation between tumor subgroups based on specific differences in metabolites.

Processed data was reviewed by both Dr Madhu Basetti and myself. Data analysis, correlation with clinical phenotype and genotype and statistical testing was performed independently.

6.34 Liquid chromatography mass spectroscopy (LC-MS):

LC-MS was performed on 20 glioma samples for more sensitive quantification of L- and D-isoforms of the 2-hydroxyglutarate (2-HGA) metabolite. This analysis was performed by Kate Donoghue and colleagues at the Bioanalytical Core of CRUK, Cambridge Institute.
All samples were homogenised using water and a 20 μL aliquot of each sample was extracted and derivatised according to the protocol in Table 6.1. Calibration standards and quality control indices were within the core facility’s acceptance criteria of ± 20%, using a calibration range of 0.25 -100 μM for both L- and D-HGA. The sample results were corrected for any dilution used to homogenise the samples, and reported in terms of μmol/kg or nmol/g of tissue to three significant figures, applying the assumption that 1 kg tissue ≈ 1 L water.

**Table 6.1:** Derivatising protocol for LC-MS performed by colleagues at Bioanalytical Core of CRUK

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Function</th>
<th>Preparation</th>
<th>Exp. Period and storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacetyl-L-tartaric anhydride (DATAN) 50g/L in dichloromethane : acetic acid (4:1)</td>
<td>Derivatising reagent</td>
<td>0.25 g (weighed in powder cabinet) of DATAN added to 4 mL of dichloromethane and 1 mL of acetic acid</td>
<td>Prepared fresh on day in fume cupboard</td>
</tr>
</tbody>
</table>

**6.35 Statistical analysis:**

I performed statistical analysis using MedCalc (version 18.2.1). A mean and standard deviation was calculated for all continuous variables. The non-parametric Spearman test was applied to investigate the correlation between metabolites and the Mann-Whitney U test was
performed to investigate the difference in the mean concentration of metabolites between groups. The receiver operating characteristic (ROC) curves were used to determine the most sensitive and specific cut off value for succinate to differentiate between SDH-deficient and non-SDH-deficient tumours and to identify a mean concentration of other metabolites, which may differentiate between SDH mutated and non-mutated tumours.

6.36 SDHB Immunohistochemistry

SDHB immunohistochemistry (IH) was performed (according to the methodologies outlined in chapter 3) on 4µm sections of FFPE tumour tissue, when available and SDHB IH was interpreted by an experienced pathologist (Dr Olivier Giger or Dr Alison Marker) and myself.
6.4 Results

6.41 Genotype and clinical phenotype of cohort of patients with GIST and PPGL

A total of 40 cases were included in the initial analysis with either a histological diagnosis of PPGL (n=32) or wild-type GIST (n=8) and additional recruitment was restricted by the availability of fresh frozen tumour samples. A pathogenic germline variant was identified in 11/38 (29%) patients tested (Table 6.2), two patients were not tested due to their personal preference. A pathogenic germline $SDHx$ variant (4 patients with a variant in $SDHB$, two in $SDHA$ and two in $SDHC$) was identified in 8 patients, two patients were diagnosed with a pathogenic germline $VHL$ variant and a single patient had a pathogenic $RET$ variant. One further patient with a metastatic wtGIST was subsequently diagnosed with somatic epigenetic silencing of the $SDHC$ gene in the tumour (case HRMAS39). Metastatic disease was identified in 5/40 (12.5%) patients and in all cases metabolomics analysis was performed on the primary tumour.
Table 6.2 Genotype and clinical phenotype of cohort

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Tumour phenotype</th>
<th>Metastatic disease</th>
<th>SDHB IH</th>
<th>Pathogenic germline variant</th>
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<td>SDHC epimutation</td>
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<td>GIST</td>
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<td>Loss</td>
<td>SDHC c.380A&gt;G (p.His127Arg)</td>
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6.42 Succinate detection ex vivo

On visual inspection, the $^1\text{H}$ NMR spectra obtained from the 40 tumours was of good quality and succinate, when detectable, was visualized as a peak at 2.4ppm on the spectra (Figure 6.11A). A succinate peak was noted as being detectable if the standard deviation of the fitted succinate peak on LCModel was <5% and based on the subjective visualization of a succinate peak at 2.4ppm on the 1D spectra by Dr Madhu Basetti and myself. According to these criteria a succinate peak was correctly identified in all 8 patients with a pathogenic germline $SDHx$ variant and succinate accumulation was also identified in the tumour of the patient with the somatic $SDHC$ epimutation. The mean succinate concentration was 15-fold higher in the $SDH$ mutated/epimutated tumours (mean succinate 4.7mM SD+3.13) compared to the $SDH$ wild-type tumours (mean succinate 0.31mM, SD+0.12, P=0.0066). No detectable succinate peak was identified in any of the sporadic tumours or tumours caused by a mutation in non-$SDHx$ predisposition gene (e.g. $VHL$ or $RET$). Detection of a succinate peak correlated with loss of SDHB protein expression on SDHB IH for 6/6 $SDHx$ mutated cases, for whom FFPE tissue was available. A succinate peak was detected in the two cases with a truncating germline $SDHA$ (c.91C>T p.Arg31Ter) variant, providing a further line of evidence to support pathogenicity of this germline variant. Furthermore, one of these cases (HRMAS38), had initially declined routine clinical genetic testing on the basis of his low risk of genetic predisposition, as he presented with an adrenal phaeochromocytoma at the age of 68 years. However metabolomic analysis demonstrated a succinate peak (concentration 1.37 mM) and SDHB IH later confirmed loss of SDHB protein expression in keeping with SDH deficiency. As a result, germline genetic testing was performed and a truncating $SDHA$ variant (c.91C>T p.Arg31Ter) was subsequently confirmed.
**Figure 6.11:** Figure A depicts the detection of the metabolite succinate at 2.4ppm on a spectra obtained by HRMAS $^1$H NMR spectroscopy from the tumour tissue of two patients with pathogenic $SDHx$ variants and no detectable succinate peak at the same frequency on the spectra in three tumours from patients without a germline $SDHx$ variant. Figure B shows the statistically significant difference in succinate concentration (mM) in $SDHx$ mutated tumours compared to $SDHx$ wild type tumours.
**Figure 6.12:** Figure A shows a ROC curve analysis demonstrating the optimal succinate concentration to differentiate $SDHx$ mutated versus non $SDHx$ mutated tumours, calculated as $>0.78$ mM. Figure B demonstrates the HRMAS $^1$H NMR spectra from a tumour without a germline variant in $SDHx$ and spectra from tumours with different $SDH$ subunit pathogenic variants, showing variation in succinate peak height at 2.4ppm on the spectra.

![Figure 6.12](image)

Interestingly, although the sample size of $SDHx$ mutated tumours was small (n=8), a difference in the size of the succinate peaks between different $SDHx$ pathogenic subunit variants was noted on subjective visualisation of the spectra (Figure 6.12) and quantification of succinate concentrations. In particular the concentration of succinate was noted to be higher in tumours with pathogenic $SDHC$ (mean succinate 5.66 mM, SD 4.41) and $SDHB$ (mean succinate 5.29mM, SD 3.8) gene variants compared to $SDHA$ (mean succinate 1.25mM, SD 0.10). However, the difference in succinate concentration between $SDHA$, $SDHB$ and $SDHC$ subunit variants did not reach statistical significance using a one way Anova test (P=0.4). No significant difference in the succinate concentration was detected in $SDHx$-mutated PPGL compared to $SDHx$-mutated GIST (p=0.8), although this direct comparison of metabolomic profiles is complicated by the genetic heterogeneity of the two tumour types.
Finally, the optimal concentration cut-off for succinate to differentiate SDHx mutated from non-SDHx mutated tumours was performed using receiver operating characteristic (ROC) curves and calculated as >0.78mM (sensitivity and specificity 100%, Figure 6.12A).

6.43 Untargeted metabolomic profiling of SDH deficient tumours

The spectra obtained at HRMAS $^1$H NMR spectroscopy provided data on the detection and quantification of 30 different metabolites in the 40 tumours analysed. In addition to the statistically significant difference in succinate concentration, further significant differences were noted in the untargeted metabolomic profiles of the SDHx mutated compared to the non-SDHx mutated tumours. Notably, significantly lower concentrations of glutamate, aspartate, leucine, valine, alanine and choline containing compounds (PCh and GPC), indicating impaired amino-acid and membrane phospholipid metabolism, were detected in the SDHx-mutated tumours compared to the non-SDHx-mutated tumours. A significantly lower concentration of lactate was also noted in the SDHx mutated tumours, but this was not uniform across all 40 tumours analysed and, as lactate levels can be altered by increased anaerobic glycolysis during tumour retrieval and storage conditions, the altered lactate levels cannot unequivocally be attributed to differences in tumour metabolism between the two SDHx variant status groups. Interestingly, I detected a statistically significant difference in glutamine concentration between the two groups but the concentration was lower in the SDHx -mutated tumours (mean 1.76mM, SD1.2) compared to the non-SDHx mutated tumours (mean 2.65mM, SD 2.47) (P=0.004), which is the opposite of what was found in other metabolomic studies (260). ROC curve analysis for those metabolites that were detected to be significantly different in the SDH mutated tumours was performed and did not demonstrate a cut off that yielded 100% sensitivity and specificity for differentiating SDHx
from non SDHx mutated tumours (in contrast to that for succinate). Aspartate was identified as the next most sensitive (100%) and specific (81%) metabolite to succinate, at a concentration of < 2.41mM on ROC curve analysis, for differentiating SDH deficient from SDH preserved tumours. (Figure 6.15).

**6.44 Unmasking of a somatic IDH1 mutation by untargeted metabolomics profiling**

Case HRMAS22, presented with an extra adrenal abdominal paraganglioma at the age of 67 years (Figure 6.14C). Germline genetic testing at presentation did not identify a pathogenic genetic variant. Metabolomics analysis did not identify a succinate peak and there was preservation of the SDHB protein expression on tumour immunohistochemistry. However, a peak was identified at 4.02ppm on the spectra (Figure 6.14A) and this was consistent with the presence of the metabolite 2-hydroxyglutarate (2HG) (Figure 6.14B). Normally the isocitrate dehydrogenase enzyme (IDH) functions in the citric acid cycle and converts isocitrate to α-ketoglutarate. However mono-allelic pathogenic missense variants in the genes IDH1 and IDH2 can result in the neomorphic production of 2HG, and these pathogenic variants and resulting altered metabolome is most commonly encountered in glioma tumours (264). Though a pathogenic somatic IDH1 variant has been reported previously in a single case of PPGL (265), to my knowledge, 2HG accumulation has not been reported in PPGL. In order to confirm this apparent novel finding on 1D spectra acquisition, two-dimensional (2D) homonuclear (1H-1H) NMR experiments were also performed by Dr Madhu Basetti, to validate the 1D finding and 2HG detection was confirmed on 2D spectra acquisition (Figure 6.14D). Subsequent sequencing of tumour DNA, confirmed a somatic pathogenic IDH1 (c.394C>T p.Arg132Cys) variant accounting for the 2HG accumulation detected in this sample.
Figure 6.14: Figure A demonstrates the 1D spectra acquired by HRMAS $^1$H NMR spectroscopy from tumour sample HRMAS22 revealing a peak at 4.02ppm. Figure B shows the exemplar spectra expected with 2HG detection, figure C illustrates the large abdominal paraganglioma, highlighted by the red arrow in case HRMAS22 and finally figure D represents a 2D spectra confirming the detection of 2HG in this tumour sample as illustrated by the red box.
6.45 Measurement of 2-Hydroxyglutarate (2HG) in IDH1 mutant glioma samples

In addition to the 40 (PPGL and GIST) tumours analysed initially, targeted and untargeted metabolomics profiling was performed on fresh frozen glioma samples in order to evaluate the detection of 2HG by HRMAS $^1$H NMR spectroscopy and to compare the metabolomics profile of IDH1 mutated tumours with SDHx mutated tumours. Due to limitations in available fresh frozen tumour samples, 20 glioma samples were analysed by both HRMAS $^1$H NMR spectroscopy and LC-MS and an additional 15 tumour samples (total n=35) were analysed by LC-MS alone.

The metabolite 2HG exists in two isoforms: L-2-hydroxyglutarate acid and D-2-hydroxyglutarate in human tissue but it is the D-isoform which accumulates due to gain-of-function oncogenic IDH1 mutations. 2HG is detected on $^1$H NMR spectroscopy due to the non-exchangeable protons provided by the five carbon atoms, which make up the molecular structure of 2HG. The spectral locations of the non-exchangeable protons of 2HG are 4.02ppm (H2), 1.83ppm and 1.98ppm (H3) and 2.25ppm and 2.29ppm (H4) (Figure 6.15).

IDH1 status of the 35 glioma tumour samples was determined by tumour sequencing (Table 6.3). The majority of tumours (32/35, 91%) harboured the most common pathogenic IDH1 variant (c.395G>A p.Arg132His) (266), two glioma tumour samples did not have a detectable variant in IDH1 or IDH2 and a single tumour sample had the less common pathogenic IDH1 missense variant (c.394C>T p.Arg132Cys). The average read depth across the IDH1 and IDH2 hot spot regions was 2,056 reads. The mean percentage of reads supporting the variant allele versus the reference allele in those tumours identified with a pathogenic IDH1 variant was 30%, indicating that 2HG should be detectable in these tumours, as neomorphic 2HG production requires mono-allelic loss compared to the bi allelic loss necessary for SDHx mutations to cause succinate accumulation.
**Figure 6.15:** Figure provided by Dr Madhu Basetti showing the spectral locations of the non-exchangeable protons (H2, H3, and H4) of 2HG.

In order to validate 2HG detection, all 20 samples were analyzed by both 1D HRMAS $^1$H NMR spectroscopy and 2D homonuclear ($^1$H-$^1$H) NMR spectroscopy and 2HG detection by HRMAS $^1$H NMR spectroscopy was defined as confident detection of a 2HG peak on both 1D and 2D spectra and a 2HG peak fitting of <20% SD on LCModel.

2HG was reliably detected by HRMAS $^1$H NMR spectroscopy in 6/18 (33%) *IDH1* mutant glioma samples (Figure 6.16), compared to all 33/35 (100%) samples using LC-MS. The mean concentration of 2HG in the 33/35 *IDH1* mutated tumours analysed by LC-MS was 1.93mM (SD 1.3). The mean concentration of 2HG in the six samples with reliable 2HG detection by HRMAS $^1$H NMR spectroscopy was (2.9mM, SD1.3) and the concentration of 2HG was >1mM in all six samples suggesting that this concentration is the lowest detection limit for NMR spectroscopy. The mean concentration of the two *IDH1* wild type tumours was (0.004mM) and almost 500 fold less than that of the *IDH1* mutant tumours.
Table 6.3: Pathogenic IDH1 variants identified in the 35 glioma samples sequenced and the % of reads supporting the variant allele in each case

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<th>Case ID</th>
<th>Mutation</th>
<th>Reads supporting variant allele and (% reads supporting variant /total reads)</th>
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<tr>
<td>HRMAS 43</td>
<td>IDH1 c.395G&gt;A (p.Arg132His)</td>
<td>415 (43%)</td>
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<td>HRMAS 44</td>
<td>IDH1 c.395G&gt;A (p.Arg132His)</td>
<td>486 (36%)</td>
</tr>
<tr>
<td>HRMAS 45</td>
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<td>870 (37%)</td>
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<td>870 (37%)</td>
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<td>717 (40%)</td>
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<td>IDH1 c.395G&gt;A (p.Arg132His)</td>
<td>894 (33%)</td>
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</table>
Figure 6.16: Figure A demonstrates 2HG peaks detected in sample HRMAS 51 in red and a standard 2HG sample peaks in black on 1D spectra acquisition by HRMAS $^1$H NMR spectroscopy, Figure B shows confirmation of 2HG detection on 2D spectra acquisition and figure C illustrates 2HG detection on LC-MS of the same sample. Figure D shows the spectra obtained for sample HRMAS46 without a detectable IDH1 variant, with only a very small peak of D-2HG detected by LC-MS.
A review of the sequencing data of the 35 glioma revealed that 12/35 (34%) \textit{IDH1} mutant glioma samples had a co-existing pathogenic somatic \textit{TP53} variant with a variant allele frequency greater than 10%. Coexisting \textit{TP53} and \textit{IDH1} variants is a well described molecular profile in diffuse astrocytoma (267). \textit{IDH1} variants have been identified as the early molecular driver in infiltrating gliomas and studies suggest that subsequent acquisition of pathogenic \textit{TP53} variants is associated with the progression to diffuse astrocytoma formation (267). Therefore I performed additional analysis to determine if there was a difference in the 2HG concentration (measured by LC-MS) between those glioma samples harbouring both \textit{IDH1} and \textit{TP53} variants versus those with \textit{IDH1} variants alone. The difference in the mean 2HG concentration was 1056.4umol/kg (1966.6umol/kg versus 910.2umol/kg), but this difference did not reach statistical significance (P=0.0884) (Figure 6.17).
Table 6.4: *IDH1* mutant glioma samples with co-existing *TP53* variants

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Pathogenic <em>IDH1</em> variants</th>
<th>Pathogenic <em>TP53</em> variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRMAS 42</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.817C&gt;T (p.Arg273Cys)</td>
</tr>
<tr>
<td>HRMAS 43</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.1010G&gt;T (p.Arg337Leu)</td>
</tr>
<tr>
<td>HRMAS 46</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.716A&gt;C (p.As239Thr)</td>
</tr>
<tr>
<td>HRMAS 51</td>
<td>c.394C&gt;T (p.Arg132Cys)</td>
<td>c.742C&gt;G (p.Arg248Gly)</td>
</tr>
<tr>
<td>HRMAS 52</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.85G&gt;A (p.Ala29Thr)</td>
</tr>
<tr>
<td>HRMAS 55</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.523C&gt;G (p.Arg175Gly)</td>
</tr>
<tr>
<td>HRMAS 57</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.817C&gt;T (p.Arg273Cys)</td>
</tr>
<tr>
<td>HRMAS 58</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.524G&gt;A (p.Arg175His)</td>
</tr>
<tr>
<td>HRMAS 71</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.743G&gt;A (p.Arg248Gln)</td>
</tr>
<tr>
<td>HRMAS 72</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.844C&gt;G (p.Arg282Gly)</td>
</tr>
<tr>
<td>HRMAS 74</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.722C&gt;T (p.Ser241Phe)</td>
</tr>
<tr>
<td>HRMAS 75</td>
<td>c.395G&gt;A (p.Arg132His)</td>
<td>c.459C&gt;T (p.Pro152Pro)</td>
</tr>
</tbody>
</table>

Figure 6.17: Mean 2HG concentration in glioma samples with *IDH1+ / TP53+* variants versus tumours with *IDH1+ / TP53-* variants
Comparing the metabolomics profiles of SDH deficient and IDH1 mutant tumours

The hypothesis for this analysis was that that both pathogenic SDHx and IDH1 variants result in a truncated citric acid cycle and inhibit alpha ketoglutarate dependant di-oxygenase enzyme reactions; therefore I postulated that the metabolomics profile associated with both genetic variants may demonstrate significant overlap. This analysis was limited by an inability to control for the heterogeneity of different tumour types and their respective tissue specific metabolome.

The data analysis technique; orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to identify a difference in the metabolomics profile of the IDH1 versus SDHx-mutated tumours. A clear separation was noted between the groups with the metabolites succinate (higher in the SDHx group compared to IDH1) and phosphocreatine (higher in IDH1 compared to SDHx group) showing the greatest difference.

Next, I compared those metabolites that were significantly different in the SDHx versus non-SDHx mutated tumours. There was a significantly lower aspartate (p=0.0002), glutamine (p=0.001), glutamate (p=0.0014) in the SDHx mutated tumours compared to the IDH1 mutated tumours. No difference in the concentration of the branch chain amino acids, was detected between the two groups (alanine (p=0.21), valine (p=0.61)).

Overall, the metabolomics profiles of the SDHx and IDH1 mutant tumours was noted to be different with the only suggestion of a potential overlap observed for the branch chain amino acids.
6.5 Discussion

6.51 Clinical utility of ex vivo metabolomics analysis using HRMAS in GIST and PPGL

This study has demonstrated that HRMAS $^1$H NMR spectroscopy is an effective tool for the detection of succinate *ex-vivo* and that quantification of succinate concentration (>0.78Mm) can be applied clinically to differentiate between $SDHx$ mutated and non-$SDHx$ mutated tumours, including those tumours caused by pathogenic variants in other predisposition genes (e.g. *VHL* and *RET*). In particular the application of succinate as a single metabolomic biomarker demonstrated clinical application in the detection of SDH-deficiency in a less typical phenotype in case HRMAS38 and prompted molecular genetic testing and the discovery of a pathogenic germline variant in $SDHA$ (c.91C>T p.Arg31Ter). The detection of excess succinate accumulation in two cases of $SDHA$ mutated tumours is also highly relevant as my previous analysis has demonstrated that false negative results using SDHB IH are more common with $SDHA$ and $SDHD$ mutated tumours, as was demonstrated in chapter 3 (case#6) in a patient with the same truncating $SDHA$ variant (c.91C>T p.Arg31Ter) as the two tumours analyzed in this study, with evidence of succinate accumulation.

Although no case harboring a variant of uncertain significance was included in this study, it would seem that metabolomic analysis is a very sensitive functional test for the verification of $SDHx$ variant pathogenicity as it provides valuable information on the SDH enzyme function and is likely to have particular application as an adjunct to SDHB IH for confirming novel $SDHx$ missense variant pathogenicity.

Importantly, I included wtGIST tumours in this study and demonstrated that succinate accumulation can also be detected in SDH deficient wtGIST *ex-vivo*, using HRMAS. This study similar to one previous study (260) has demonstrated that detection of succinate accumulation can identify SDH deficiency due to epigenetic silencing of the $SDHC$ gene.
Analysis of a wtGIST sample from case HRMAS39, revealed a succinate peak at 2.4ppm similar to other cases harboring an SDHx pathogenic variant and succinate concentration was quantified as 2.41mM. The diagnosis of an SDHC epimutation was confirmed by pyrosequencing of tumour DNA, confirming hypermethylation at the promotor region of the SDHC gene. Therefore targeted metabolomic analysis of succinate by HRMAS can reliably detect SDH-deficiency due to both genetic and epigenetic SDHx variants in PPGL and GIST.

An intriguing observation in this study was the difference in the mean concentration of succinate detected between tumours with different SDHx subunit gene variants. This observation did not reach statistical significance and sample size was small but it would be interesting to investigate in a larger cohort as it might provide insights into the differences in clinical phenotypes between SDH-subunits e.g. SDHB variants having a much higher risk of malignant potential compared to SDHA and higher penetrance with germline mutations in SDHC than SDHA. Differences in succinate concentrations may also point towards tissue specific threshold for oncogenesis, as SDHA variants are more commonly associated with GIST and SDHB with RCC (87).

In this study, untargeted metabolomic analysis yielded the unexpected finding of 2HG accumulation in an abdominal paraganglioma tumour sample (HRMAS22). The detection of this oncometabolite prompted additional tumour sequencing and the discovery of a pathogenic somatic IDH1 variant (c.394C>T p.Arg132Cys). To my knowledge, this is the second case of an IDH1 mutated PPGL reported to date and the first case describing 2HG accumulation in a PPGL tumour. This case highlights the application of untargeted metabolomic analysis for the detection of occult genetic drivers and demonstrates that metabolomic profiling can be employed for the detection and validation of germline and somatic gene mutations in metabolically driven tumours.
6.52 Metabolic reprogramming in SDH mutated tumours

The untargeted metabolomics profiling also demonstrated that SDH deficient tumours had a specific metabolomics fingerprint compared to non SDHx mutated tumours, with lower concentrations of metabolites involved in amino acid and membrane phospholipid metabolism. The hypothesis for the reduction in the two essential amino acids; aspartate and glutamate in SDH mutated tumours is believed to be caused by the truncated citric acid cycle coupled with the inhibition of α-ketoglutarate. However alternative hypotheses include; reduced expression of glutamate-aspartate membrane transporters (aspartate-glutamate carrier 1, AGC1) in SDH deficient tumours (268) and increased glutamate release into the extracellular space to promote cell proliferation and invasion, as has been demonstrated in glioblastoma (269). A lower concentration of glycine has also been demonstrated in cancer cells and attributed to excess glycine consumption to fuel cellular proliferation (270) and therefore the significantly lower glycine in SDHx mutated tumours in this study is noteworthy.

Perhaps more pertinent than the cause of the unique metabolomics fingerprint in SDH deficient tumours, is the mechanisms by which the apparent deficiency in key metabolites is overcome by these tumours. Metabolic re-programming is a common feature of cancer cells in order to prioritise cellular proliferation and is typically characterised by an increased consumption of glucose and glutamine and increased glycolytic substrate production (270). However, metabolomics profiling of SDH deficient tumours in this study and others (260) has revealed that the metabolic reprogramming extends further into the central carbon metabolism, affecting amino acid intermediates. Cancer cells rely on aspartate for DNA synthesis and to enable cellular proliferation, therefore it is intriguing that SDHx-mutated tumours have a lower aspartate and previous studies have demonstrated that SDH-deficient tumours must become reliant on alternate pathways and upregulation of enzymes such as the
pyruvate carboxylase enzyme, to convert pyruvate to oxaloacetate and aspartate (271), in order to ensure the continual generation of metabolites necessary for nucleotide production, biomass and cellular proliferation. This reliance on the carboxylation of pyruvate in \( SDHx \) mutated cells might explain why lower concentrations of the branched chain amino acids; alanine, valine and leucine were detected in \( SDHx \) mutated tumours in this study, suggesting perhaps that the transamination of pyruvate is sacrificed in favour of carboxylation to oxaloacetate in SDH deficient cells (Figure 6.20). It is also notable that a rare metabolic deficiency of pyruvate carboxylase is associated with the inverse metabolomic profile to that identified in our SDH deficient tumours, namely a raised alanine (272). The reliance on alternate metabolic pathways suggests a metabolic vulnerability of SDH-deficient tumours and offers an opportunity to further investigate how this metabolic vulnerability could be targeted for therapeutic benefit.
6.20: This figure shows some of the citric acid cycle intermediate metabolites (in blue) that were significantly different in SDH mutated compared to non-SDH mutated tumours in this study.

![Citric Acid Cycle Diagram]

6.53 Limitations of HRMAS $^1$H NMR spectroscopy

While metabolomics profiling using HRMAS $^1$H NMR spectroscopy can inform on steady state changes in metabolite concentration and enables tumour stratification based on specific metabolic signatures, it does not provide information on enzyme flux, therefore a combined approach with techniques such as isotope labelling which provide insight into biochemical flux, is crucial for therapeutic target exploration (271). This study also revealed a limitation of HRMAS $^1$H NMR spectroscopy in the detection of 2HG. There are also overlapping signals from nearby metabolites on the spectra such as glutamate and glutamine and these factors can impact on the sensitivity of 2HG detection by NMR spectroscopy. Therefore the
results from this study would support the use of LC-MS as the superior technique for the targeted metabolomic profiling of 2HG.

Another limitation of *ex vivo* metabolomics analysis using HRMAS $^1$H NMR spectroscopy or $^1$LC-MS, is the limited availability of fresh frozen tumour. Formalin fixed paraffin embedded (FFPE) tumour samples are routinely used for histological diagnosis but metabolomic analysis using FFPE tissue has been limited owing to concern about the effect of tissue processing on metabolite content. Recently a new protocol for the *ex vivo* metabolomic analysis of FFPE samples was reported, namely; a high-mass-resolution matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance (MALDI) mass spectrometry imaging technique (273). The metabolomics profiles analysed using this technique were compared to that of fresh frozen tumour samples and demonstrated that 70% of the metabolites were conserved and detectable in the FFPE samples using MALDI mass spectrometry (273).

However, even when fresh frozen tumour is available, or techniques optimised for FFPE tissue analysis, the variance in the metabolomics profile introduced by the surgical procedure and storage conditions, is difficult to control for. One option for overcoming this limitation of *ex-vivo* analysis, is to move the analysis to the *in-vivo* setting, as has been performed for *IDH1* mutant tumours (274).
Conclusion:

This study has identified that HRMAS $^1$H NMR spectroscopy can detect succinate accumulation ex vivo and that a succinate concentration $>$0.78mM can differentiate $SDHx$ mutated tumours from non $SDHx$ mutated tumours with 100% sensitivity and specificity. This study has highlighted a number of key clinical applications of this technique including: i) the diagnosis of an SDH deficient tumour, which can trigger genetic testing, ii) as an adjunct functional tool to SDHB IH for assessing $SDH$ variant pathogenicity and iii) for the identification of somatic $SDHx$ pathogenic variants or epimutations. Furthermore untargeted metabolomics analysis revealed an occult pathogenic somatic $IDH1$ variant and identified a specific metabolomics profile unique to $SDHx$ mutated tumours. This study also identified important limitations of HRMAS $^1$H NMR spectroscopy, including the low detection rate of 2HG using this technique. In the future, a combined approach of metabolomics profiling and isotope labelling experiments both $ex$ $vivo$ and $in$ $vivo$, may further enlighten therapeutic strategies in $SDHx$ mutated tumours.
Chapter 7

Translating *in vivo* metabolome analysis of SDH deficient tumours into clinical utility

DECLARATION: Findings described in this chapter have been published previously in:

Chapter 7: Translating *in vivo* metabolome analysis of SDH deficient tumours into clinical utility

7.1 Introduction

7.11 MRI spectroscopy

7.12 Molecular imaging in PPGL

7.13 Molecular imaging in wild type (wt)GIST

7.14 In vivo analysis of citric acid cycle ‘oncometabolites’

7.2 Aims

7.3 Methods

7.31 Patient selection

7.32 MRI spectroscopy (1H-MRS) analysis

7.33 1H-MRS data acquisition

7.34 Germline genetic analysis

7.35 SDHB Immunohistochemistry

7.36 SDHC promoter methylation analysis

7.37 Statistical analysis

7.4 Results

7.41 Patients and clinical phenotype

7.42 Genotype

7.43 1H-MRS succinate analysis

7.5 Discussion

7.51 Translational applications of in vivo metabolomic analysis using 1H-MRS

7.52 Lessons learned regarding optimisation and limitations of in vivo metabolomic analysis using 1H-MRS in SDH deficient tumours

7.53 Future applications of in vivo metabolomics analysis

7.6 Conclusion
7.1 Introduction

As ex-vivo metabolomics analysis demonstrated that succinate levels are elevated by fifteen-fold in SDH deficient tumours (including GIST and PPGL) compared to non-SDHx mutated tumours (Chapter 6), I wanted to investigate the potential clinical and translational applications of measuring succinate levels in vivo as the next step.

7.11 MR spectroscopy

Proton magnetic resonance spectroscopy ($^1$H-MRS) is a non-invasive radiological modality, which enables the in vivo assessment of a localised chemical environment (275). $^1$H-MRS is most commonly used as a complementary analytical tool to magnetic resonance imaging (MRI) in the characterisation of normal tissue, an inflammatory/infective process or a tumour. $^1$H-MRS similar to MRI acquires signal from hydrogen protons, however the signal acquired by $^1$H-MRS is from a focal or targeted region and referred to as a voxel. As in HRMAS for ex-vivo metabolomics analysis, in $^1$H-MRS each metabolite gives rise to peaks at specific chemical shifts, thus allowing metabolites to be identified based on their unique $^1$H-MRS fingerprint.

$^1$H-MRS has been applied to the study of a number of disease processes including Alzheimer’s disease (276), epilepsy (277), tuberculosis(278) and perhaps the most notable success has been demonstrated in the study of cancer including prostate (279), breast cancer (280) and brain tumours (281).

The biggest challenge for the direct translation of $^1$H-MRS imaging from the research setting into the clinical arena is the need for high quality spectra which is dependent on the optimisation of a number of factors including: homogeneity of the magnetic field, acquisition
parameters, water and fat suppression, and accuracy and objective nature of the post
processing software programs. Therefore additional studies are required to define the optimal
conditions for high quality spectral acquisition using $^1$H-MRS in new disease processes and,
importantly, to define the limitations of this imaging modality before translation into the
clinical setting is feasible.

7.12 Molecular imaging in PPGL

Imaging with $^{18}$F-fluorodeoxyglucose positron emission tomography ($^{18}$F-FDG PET) to
measure the uptake and phosphorylation of a glucose analogue to probe the increased glucose
use that occurs in many metabolically active cancers is a useful form of in vivo metabolic
imaging and has been used for the detection of primary and metastatic diseases in many
tumour types, including PPGL (282) and GIST (283) and is in widespread clinical use.
Indeed, targeted nuclear imaging has been applied as an adjunct to morphological cross
sectional imaging studies, to the diagnosis and management of PPGL for the past four
decades and the tracers used can be sub-classified based on their target into three groups; i)
catecholamine storage and synthesis ($^{123}$I-metaiodobenzylguanidine ($^{123}$I–MIBG), $^{18}$F-
fluorodopamine ($^{18}$F-FDA), and $^{18}$F-fluorodihydroxyphenylalanine ($^{18}$F-FDOPA), ii)
somatostatin receptor ($^{111}$Indium-pentetreotide and $^{68}$Ga-labeled somatostatin analog peptides
and iii) glucose metabolism ($^{18}$F-FDG)

The first tracer employed in the study of PPGL was the norepinephrine analog, $^{123}$I- MIBG,
which is taken up by the norepinephrine transporter (NET)(284). $^{123}$I-MIBG scintigraphy has
proven benefit in the localisation of adrenergic phaeochromocytoma (285), however it has
been well demonstrated that the sensitivity of $^{123}$I-MIBG scintigraphy is limited in PPGL.
Firstly, tumour de-differentiation associated with metastatic or aggressive disease can lead to
a loss of NET expression and increase the risk of false negative results using $^{123}$I-MIBG scintigraphy (286). Furthermore, mutations in $SDHx$ are associated with a higher risk of false negative results because of an associated downregulation of the NET transporter in SDH deficient tumours (287). Therefore, current recommendations advice that $^{123}$I-MIBG scintigraphy should be reserved for those cases being investigated for suitability of treatment with $^{123}$I-MIBG radionuclide therapy (208). Similarly, the tracers $^{18}$F-FDA, taken up by the NET transporter and $^{18}$F-FDOPA, taken up through the LAT amino acid transporter, showed initial promise as functional imaging tracers in PPGL (285), however these nuclear imaging tracers are now seldom used due to the availability of superior more sensitive nuclear imaging tracers such as $^{18}$F-FDG (208) and $^{68}$Gallium-DOTATATE (288).

PPGL are known to express somatostatin receptors (289) and as such the $^{111}$In-Pentetreotide, a ligand of somatostatin receptors, has demonstrated efficacy in the functional imaging of PPGL (290). In contrast to the previous category of tracers, $^{111}$In-Pentetreotide (Octreoscan) scintigraphy has demonstrated superior efficacy in localising metastatic PPGL and has proven to be a less sensitive imaging modality for the detection of primary PPGL (291). Therefore, $^{111}$In-Pentetreotide (Octreoscan) scintigraphy is reserved primarily for those patients with metastatic disease, who are being considered for treatment with peptide receptor radionuclide treatment (PRRT) with radiolabeled somatostatin analogues. More recently a new somatostatin analog ($^{68}$Ga-labeled somatostatin analog) with higher affinity for somatostatin receptor 2 (288), preferentially expressed on neuroendocrine cell and superior sensitivity when combined with PET-CT, has emerged as the preferred tracer to target somatostatin receptor expression in primary and metastatic PPGL (288). However, one of the drawbacks with $^{68}$Ga-DOTATATE PET-CT, is the poor sensitivity of this tracer for primary phaeochromocytoma, owing to the high physiological uptake of $^{68}$Ga-labeled somatostatin analogues in the healthy adrenal (292).
\(^{18}\)F-FDG PET-CT is currently recommended as the molecular imaging modality of choice for the localisation of PPGL\(^{(208)}\). Intriguingly, the sensitivity of \(^{18}\)F-FDG PET-CT also differs depending on the molecular pathways implicated in the development of PPGL, with tumours harbouring pathogenic \(SDHx\) variants demonstrating increased standard uptake values (SUV) of \(^{18}\)F- FDG \(^{(293)}\)(\(^{(294)}\)). The superior performance of \(^{18}\)F-FDG PET-CT in localising SDH-deficient PPGL, has been attributed to enhanced glucose phosphorylation by hexokinases in SDH-deficient PPGL rather than increased expression of glucose transporters \(^{(294)}\).

In addition to its role in locating PPGL, \(^{18}\)F-FDG PET CT may also have a role in evaluating therapeutic response \(^{(20)}\), although further studies are needed to better inform this potential application of \(^{18}\)F-FDG PET-CT in metastatic PPGL.

### 7.13 Molecular imaging in wild type (wt) GIST

The conventional radiological measurement of tumour response including uni- or bidimensional changes in tumor size (Response Evaluation Criteria in Solid Tumors, RECIST) are routinely applied in clinical practice, however the benefit of evaluating metabolic response using \(^{18}\)F-FDG PET-CT is well demonstrated in \(KIT\)- or \(PDGFRA\)-mutated GIST tumours following treatment with the tyrosine kinase inhibitor; imatinib \(^{(295)}\), as mesenchymal tumours such as GIST rarely demonstrate a reduction in size despite therapeutic response \(^{(296)}\). GIST exhibit high glycolytic activity and are therefore associated with increased uptake of \(^{18}\)F-FDG \(^{(295)}\). A decrease in the glycolytic metabolism of GIST in patients with a treatment response to imatinib is characterised as reduced metabolic activity on a follow up \(^{18}\)F-FDG PET-CT compared to baseline and the pivotal trial demonstrating therapeutic efficacy of imatinib in GIST, demonstrated that a SUV of <2.5 on follow up 18F-
FDG PET CT was associated with improved therapeutic response to imatinib (295). Unfortunately previous studies have not stratified patients based on molecular profiling of GIST and therefore the value of $^{18}$F-FDG PET-CT to determine therapeutic response to imatinib is not well understood for wtGIST and there is increasing evidence that wtGIST are less responsive to imatinib compared to those GIST with pathogenic somatic KIT and PDGRA variants (98). As the majority of wtGIST are SDH-deficient, it could be assumed that the mechanisms associated with increased $^{18}$F-FDG uptake in SDH-deficient PPGL, namely upregulation of hexokinases rather than glucose transporters (294), would also apply to SDH-deficient GIST and that this may mitigate any benefit of $^{18}$F-FDG PET-CT in determining therapeutic response to imatinib. However further studies are required to investigate this hypothesis. Similarly the role of functional imaging using somatostatin radiolabeled tracers in wtGIST is yet to be elucidated.

Despite ongoing advances and improving sensitivity of functional tracers and combined imaging techniques, the ability to measure individual metabolites within a tumour is beyond the scope of these tests and investigating the application of other imaging modalities such as $^1$H-MRS, may be beneficial as an alternative or complementary imaging option in patients with SDH-deficient tumours.
Figure 7.1: Functional imaging ligands in PPGL. Image adapted from (297)
7.14 In vivo analysis of citric acid cycle ‘oncometabolites’

The first in vivo analysis of an oncometabolite was the detection of 2-hydroxyglutarate with $^1$H-MRS in gliomas in patients with a somatic gain-of-function variant in the citric acid cycle enzyme, isocitrate dehydrogenase 1 ($\text{IDH1}$) (298). Genome wide sequencing studies have identified that pathogenic somatic heterozygous variants in $\text{IDH1}$ are frequently encountered in grade II-IV gliomas (299) and the detection of a somatic $\text{IDH1}$ variant provides valuable prognostic information. Studies have determined that the median survival is up to 4-fold longer for those patients with an identified pathogenic $\text{IDH1}$ variant compared to those patients with wild-type $\text{IDH1}$ gliomas (31). Therefore the potential to apply a non-invasive technique to rapidly stratify suitable patients for clinical trials and specific therapies in a tumour with a unique molecular profile, was first recognised for $\text{IDH1}$-mutant gliomas.

More recently, in vivo detection of succinate by $^1$H-MRS was reported in two small patient cohorts with SDH-deficient PPGLs (300) (301). Both of these studies focused on SDH-deficient PPGL only and neither study explored the potential role of $^1$H-MRS in monitoring therapeutic response. Both studies reported technical difficulty with smaller lesions and a technical failure rate of 37.5% in one of the studies (300). However these studies confirmed that succinate accumulation in SDH-deficient PPGL could be detected in vivo using $^1$H-MRS, but a number of questions remained unanswered, namely the role of $^1$H-MRS in monitoring therapeutic response and the optimal clinical conditions for applying $^1$H-MRS in the study of SDH-deficient tumours.
7.2 Aims

1. To investigate the role of $^1$H-MRS in detecting abnormally elevated succinate *in vivo* in patients with suspected SDH-deficient tumours and to expand the application of $^1$H-MRS to other non-PPGL SDH-deficient tumours.

2. To explore the technique of $^1$H-MRS as a potential non-invasive biomarker of treatment response.

7.3 Methods

7.31 Patient selection

This study was performed as a prospective case series, and participants were recruited from a dedicated neuroendocrine tumour clinic and a national paediatric and adult wtGIST clinic of the Cambridge University National Health Service Foundation Trust. Suitable patients were identified based on SDHx germ line status, suspicious clinical phenotype (metastatic PPGL, PGL, or wtGIST), and/or immunohistochemistry of tumour tissue showing absent SDHB immunostaining. A minimum tumour size threshold of 1.5 cm was applied for inclusion in the study. All participants provided written informed consent, and the study was approved by the Cambridge South Research Ethics Committee.

7.32 MRI spectroscopy ($^1$H-MRS) analysis

Spectral analysis was performed by Dr Mary McLean (Cancer Research UK Cambridge Institute,) and then each case was re-analysed by Dr McLean and myself, in order to interpret and correlate the spectra with the clinical and molecular data.
Both SAGE (GE Healthcare, Waukesha, WI) and LCModel (s-provencher.com) spectroscopy analysis programs were used to reconstruct, analyze, and display spectra. For each metabolite, LCModel reports both peak area and estimated uncertainty in fitting of the peak (percent standard deviation [SD]). This uncertainty measure was used to stratify the results using the following algorithm: 1) if percent SD of choline was >15%, the spectrum was discarded as a technical failure, because it was assumed that choline should be detectable in a metabolically active tumour, such that SD >15% would indicate probable data quality issues; 2) succinate detection was taken as positive if the SD was < 50% and negative if it was >50%. The succinate-to choline ratio (SCR) was quantified, the full width at half maximum height of the water peak in Hz was measured in SAGE, and recorded as an additional data quality metric. An expert spectroscopist (Dr Mary Mclean) then determined if succinate peaks were convincing or unconvincing based on data displayed both in LCModel and in SAGE.

7.33 $^1$H-MRS data acquisition

$^1$H-MRS data acquisition was performed by Dr Mary McLean and myself. All $^1$H-MRS studies were performed on a 3T-MRI system (MR750, GE Healthcare, Waukesha, WI), with body coil transmission and reception coils tailored to the tumour location. Routine T1- and T2-weighted images were acquired and a single voxel sized between 2.2 and 100 ml was prescribed within a tumour. Automated adjustment of transmitter frequency and power and magnetic field homogeneity was performed on all voxels prior to acquisition. Spectra with an echo time of 144ms were acquired from the tumour with and without chemical shift selective (CHESS) water suppression pulses, using respiratory gating when the location was in the upper abdomen. The spectral acquisition time was 15-20 min on average for each patient but
the actual time and the number of averages varied depending on the location of the tumour and the respiratory rate of the patient when respiratory triggered.

7.34 Germline genetic analysis

See methods section, chapter 2.

7.35: SDHB Immunohistochemistry

SDHB immunohistochemistry (IH) was performed on 4 µm sections of formalin-fixed paraffin-embedded tissue, after appropriate selection of tissue blocks by experienced pathologists (Dr Alison Marker and Dr Olivier Giger).

7.36 SDHC promoter methylation analysis

Methods for SDHC promoter methylation analysis will be described in detail in chapter 8. In order to assess the methylation level of the SDHC promoter region DNA was amplified as previously described by Andreasson et al (302) and the methylation level assessed by pyrosequencing®.

• Primers used for SDHC promoter region amplification:

SDHC F: 5’ GAGGAGGAGATTAAAAAATTAGAAAATAAT

SDHC R: 5’ Bio-CCACTAAAATCACCTCAACAACAA

SDHC Seq: 5’ GTTATATGATATTTTAATTT
7.37 Statistical analysis

Statistical tests were performed using MedCalc (version 18.2.1). Summary statistics include mean and standard deviation for continuous variables and frequency and percentage for categorical variables.
7.4 Results

7.41: Patients and Clinical Phenotypes

Fifteen patients (six women and nine men; mean age, 40 years [range, 21 to 80 years]) were studied. Seven wtGISTs, three unilateral adrenal pheochromocytomas, three abdominal PGLs, a large left glomus HNPGL, and a non-functioning pituitary macroadenoma were examined. Nine patients (60%) had metastatic disease: six with wtGISTs, two with abdominal PGLs, and one with a unilateral phaeochromocytoma. The liver was the most common site for metastases (7/9 (77.7%) patients). Three patients had multicentric primary tumours, including patient #5, who presented with a metastatic wtGIST and was subsequently diagnosed with a 1.9cm carotid body HNPGL (Fig 7.7D); patient #9, with an abdominal PGL and a small left-sided 1.5-cm carotid HNPGL (Fig 7.2B); and patient #8, with a large left-sided glomus PGL and a 2cm prolactin-secreting pituitary adenoma (Fig 7.4). Only two patients had positive family histories (patients #2 and #6; Table 7.1). The five patients with PPGL had noradrenaline secreting tumours on plasma metanephrine testing (median 5760pmol/l, range 318-30,849pmol/l) and no patient had co-secretion of adrenaline. Three patients with extra adrenal paragangliomas (subjects #5, #8 and #9) had non-secretory tumours including negative plasma methoxytyramine levels (<75pmol/l).

7.42: Genotype

A pathogenic germline variant in an SDHx gene was identified in 9/15 (60%) of subjects: 5 in SDHB (4 missense variants and 1 truncating variant) and 4 in SDHA (1 missense and 3 truncating). Two further patients were diagnosed with a somatic SDHC epimutation.
<table>
<thead>
<tr>
<th>Case #</th>
<th>Pathogenic germline variant</th>
<th>Sex</th>
<th>Age</th>
<th>Primary tumour</th>
<th>Metastatic disease</th>
<th>Site of metastatic disease</th>
<th>Family history</th>
<th>Other primary tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>SDHC</em> epimutation</td>
<td>F</td>
<td>21</td>
<td>GIST</td>
<td>Yes</td>
<td>Liver, lung</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td><em>SDHB</em> c.268C&gt;T p.(Arg90*)</td>
<td>F</td>
<td>53</td>
<td>Abdominal PGL</td>
<td>Yes</td>
<td>Lymph nodes, bone</td>
<td>Yes-mother (GIST)</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td><em>SDHC</em> epimutation</td>
<td>F</td>
<td>25</td>
<td>GIST</td>
<td>Yes</td>
<td>Liver</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>No mutation detected</td>
<td>F</td>
<td>27</td>
<td>GIST</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td><em>SDHB</em> c.137G&gt;A p.(Arg46Gln)</td>
<td>M</td>
<td>38</td>
<td>GIST</td>
<td>Yes</td>
<td>Liver, peritoneum</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td><em>SDHB</em> c.380G&gt;T p.(Ile127Ser)</td>
<td>M</td>
<td>80</td>
<td>PA</td>
<td>No</td>
<td>NA</td>
<td>Yes nephew (PPGL)</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>No mutation detected</td>
<td>M</td>
<td>70</td>
<td>PC</td>
<td>Yes</td>
<td>Liver, bone</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td><em>SDHB</em> c.600G&gt;T p.(Trp200Cys)</td>
<td>M</td>
<td>41</td>
<td>Glomus PGL</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>Yes, PA</td>
</tr>
<tr>
<td>9</td>
<td><em>SDHB</em> c.302G&gt;A p.(Cys101Tyr)</td>
<td>M</td>
<td>26</td>
<td>Abdominal PGL</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>Carotid PGL</td>
</tr>
<tr>
<td>10</td>
<td>No mutation detected</td>
<td>M</td>
<td>23</td>
<td>PC</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td><em>SDHA</em> c.91C&gt;T p.(Arg31Ter)</td>
<td>F</td>
<td>21</td>
<td>GIST</td>
<td>Yes</td>
<td>Liver</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td><em>SDHA</em> c.1765C&gt;T p.(Arg589Trp)</td>
<td>F</td>
<td>37</td>
<td>GIST</td>
<td>Yes</td>
<td>Liver</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td><em>SDHA</em> c.91C&gt;T p.(Arg31Ter)</td>
<td>M</td>
<td>46</td>
<td>PGL</td>
<td>Yes</td>
<td>Bone</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td><em>SDHA</em> c.91C&gt;T p.(Arg31Ter)</td>
<td>M</td>
<td>24</td>
<td>GIST</td>
<td>Yes</td>
<td>Liver</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>No mutation detected</td>
<td>M</td>
<td>67</td>
<td>PC</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
7.43: $^1$H-MRS Succinate Analysis

The $^1$H-MRS characteristics of the 15 patients are listed in Table 7.2. Mean size of the tumours selected for spectral acquisition was 5.5 cm (median, 3.3 cm; range, 1.8 to 12 cm). The liver was the most common site to be assessed (n=6), but good-quality spectra were also obtained from a pituitary tumour (n=1) and PPGLs (n=5). Patients were divided into four groups according to whether a succinate tumour peak was present, a succinate tumour peak was absent, a borderline peak was detected, or technical failure prevented interpretation of the spectra.
Table 7.2: Characteristics of the 15 tumours analysed by 1H-MRS.

TF: technical failure, defined as an estimated uncertainty (%SD) >15% in automated peak fitting of Choline using LCModel. ND: not detected, NA: not applicable

<table>
<thead>
<tr>
<th>Case #</th>
<th>Tumour site analysed</th>
<th>Maximum tumour diameter</th>
<th>Succinate: Choline ratio</th>
<th>Water FWHM</th>
<th>%SD Choline</th>
<th>%SD succinate</th>
<th>Succinate detected or technical failure</th>
<th>Convincing succinate seen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liver</td>
<td>2.6cm</td>
<td>0.74</td>
<td>13</td>
<td>2</td>
<td>4</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>2</td>
<td>Lymph node</td>
<td>8cm</td>
<td>1.32</td>
<td>6</td>
<td>11</td>
<td>47</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>3</td>
<td>Liver</td>
<td>2.3cm</td>
<td>0.74</td>
<td>10</td>
<td>8</td>
<td>23</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>4</td>
<td>GIST</td>
<td>3.6cm</td>
<td>0.07</td>
<td>18</td>
<td>4</td>
<td>113</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>5</td>
<td>Liver</td>
<td>2.5cm</td>
<td>0.15</td>
<td>12</td>
<td>5</td>
<td>27</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>6</td>
<td>PA</td>
<td>1.8cm</td>
<td>ND</td>
<td>12</td>
<td>4</td>
<td>ND</td>
<td>-</td>
<td>N</td>
</tr>
<tr>
<td>7</td>
<td>PC</td>
<td>12cm</td>
<td>0.18</td>
<td>12</td>
<td>3</td>
<td>24</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>8</td>
<td>Glomus PGL</td>
<td>9cm</td>
<td>1.71</td>
<td>29</td>
<td>6</td>
<td>8</td>
<td>+</td>
<td>N</td>
</tr>
<tr>
<td>9</td>
<td>Abdominal PGL</td>
<td>8cm</td>
<td>2.49</td>
<td>12</td>
<td>7</td>
<td>9</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>10</td>
<td>PC</td>
<td>9cm</td>
<td>0.9</td>
<td>12</td>
<td>8</td>
<td>18</td>
<td>+</td>
<td>N</td>
</tr>
<tr>
<td>11</td>
<td>Liver</td>
<td>3cm</td>
<td>0.80</td>
<td>8</td>
<td>9</td>
<td>20</td>
<td>+</td>
<td>Y</td>
</tr>
<tr>
<td>12</td>
<td>Liver</td>
<td>2.2cm</td>
<td>ND</td>
<td>18</td>
<td>16</td>
<td>NA</td>
<td>TF</td>
<td>N</td>
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<tr>
<td>13</td>
<td>Rib metastases</td>
<td>1.8cm</td>
<td>ND</td>
<td>42</td>
<td>36</td>
<td>18</td>
<td>TF</td>
<td>N</td>
</tr>
<tr>
<td>14</td>
<td>Liver</td>
<td>2.3cm</td>
<td>ND</td>
<td>15</td>
<td>32</td>
<td>27</td>
<td>TF</td>
<td>N</td>
</tr>
<tr>
<td>15</td>
<td>PC</td>
<td>5cm</td>
<td>ND</td>
<td>19</td>
<td>73</td>
<td>NA</td>
<td>TF</td>
<td>N</td>
</tr>
</tbody>
</table>
i) Succinate peak detected

Succinate was detected at 2.4ppm in six patients (50%). The mean succinate-to-choline ratio (SCR) in these patients was 1.3 (SD±0.71), and the mean tumour size in these six patients with reliable succinate peak detection was 4.8cm (SD±2.94cm; range, 2.3 to 9cm). The in vivo detection of succinate on 1H-MRS correlated with tumour SDH-deficiency; four of the six patients had pathogenic germline SDHx variants (Table 7.1), and loss of SDHB expression on immunohistochemistry and a somatic SDHC epimutation were detected in two of the six patients (Fig7.3).

ii) Borderline succinate peak detected

A borderline succinate peak was detected in two patients. Patient #8, with a pathogenic germline SDHB variant (c.600G>T p.Trp200Cys) and a glomus HNPGL, demonstrated an SCR of 1.19; however, the line width (29Hz) was so broad as a result of the proximity of metallic dental work that the peak assignments were not reliable (Figure 7.4). Patient #7, with a metastatic phaeochromocytoma and no detectable germ line SDHx variant, demonstrated an SCR of 0.18, but LCModel detected a small succinate peak at 2.4ppm; this patient did not undergo surgery or diagnostic biopsy, and therefore, no tissue was available for further analysis; we classified this case as borderline.
**Figure 7.2:** (A) T$_2$-weighted MRI showing a large non-secretory abdominal paraganglioma from case 9 (arrow). (B) Axial fused 18F-FDG PET/CT image. (C) 1H-MR spectra demonstrating a succinate peak at 2.4 ppm. The corresponding coronal maximum intensity projection (MIP) PET image demonstrates a synchronous left sided carotid paraganglioma (B). Spectra acquired by High Resolution Magic Angle Spinning (HR-MAS) *in vitro* on the paraganglioma tumour sample, again confirming a succinate peak at 2.4 ppm (D).
**Figure 7.3:** (A): $T_2$-weighted MR image from case #1 and (B) $T_1$-weighted image from case #3 demonstrating liver metastases from which spectra were acquired in the locations indicated by the white arrows. (C-D) show the spectra from case #1 and case #3 demonstrating a succinate peak at 2.4ppm. (E-F) demonstrate hypermethylation of the promoter region of the $SDHC$ gene in tumour DNA from cases #1 and #3, confirming a somatic $SDHC$ epimutation: 55% mean methylation in case 1 and 75% mean methylation in case #3.
Figure 7.4: (A) Coronal MRI image of a large left sided glomus paraganglioma from case #8 demonstrated by the white arrow. (B) Spectra processed with LCModel from the same patient showing a broad unreliable peak at 2.4 ppm, which was not convincing for succinate.

Figure 7.5: (A) Coronal T1-weighted MRI demonstrating a remnant pituitary adenoma in case #6 (white arrow). (B) Spectra acquired from the pituitary tumour at 1H-MRS, with evidence of choline detection but no succinate. (C) SDHB IHC demonstrating preservation of the SDHB protein performed on a section of tumour tissue debulked from the pituitary tumour.
iii) No succinate peak

No succinate peak was detected in three patients. Patient #4 had a metastatic wtGIST with no detectable germline SDHx variant and preserved SDHB protein expression in the tumour tissue; choline was confidently fitted on LCModel, but no succinate was seen. Patient #6 demonstrated a good-quality spectrum from the remnant pituitary adenoma; choline was detected on LCModel and SAGE processing, but no succinate was detected, and this finding was consistent with the preservation of SDHB protein expression in the pituitary tumour by immunohistochemistry (Fig 7.5). Patient #10 had no detectable germline SDHx variant and preserved SDHB protein expression in the tumour tissue; choline was detected in the tumour on 1H-MRS, but succinate was not detected.

iv) Technical failure

Technical failure occurred in four patients (26%). Patient #12 demonstrated no reliable detection of succinate or choline because of motion artefact and a low signal-to-noise ratio (SNR), which probably resulted from inconsistent breathing, because the voxel was at the edge of the liver. A small rib metastasis was imaged in patient #13, but only a pure lipid spectrum was obtained from this challenging location. A metastasis on the edge of the liver was imaged in patient #14, where again inconsistent respiration probably led to displacement of the voxel into adjacent adipose tissue. Finally, patient #15 had a unilateral phaeochromocytoma with a large volume of blood, the paramagnetic properties of which may have affected acquisition, leading to a low SNR.
v) Sequential $^1$H-MRS Succinate Analysis

Patient #2 with a metastatic PGL to the lung, bone, and lymph node and a pathogenic germline $SDHB$ variant (c.268C>T p.Arg90Ter) underwent $^1$H-MRS on a large pelvic nodal metastasis before treatment with four cycles of lutetium-177–labeled peptide receptor radionuclide therapy. Succinate and choline peaks were detected, with an SCR of 1.32 (Figs 7.6A and B). After four cycles of treatment, a repeat $^1$H-MRS examination on the same pelvic nodal metastases revealed a choline peak but no succinate peak (Fig 7.6C). Although the magnetic resonance imaging features of the metastatic lesions were unchanged pre- and post-treatment, the loss of a succinate peak was correlated with a reduction in plasma normetanephrine levels (from 1,861 to 1,193 pmol/L) and tumour avidity on $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) positron emission tomography (PET)/computed tomography (CT; standardized uptake value: pretreatment, 16.1; post-treatment, 9.3; Figs 7.6D to 7.6F).

The detection of choline on the acquired spectra both before and after treatment indicates that tumor necrosis is unlikely to account for the absent succinate peak post-treatment.
Figure 7.6: (A) Axial T2-weighted MRI image of a retroperitoneal nodal metastases from case 2 (arrow). (B) Spectra acquired before treatment illustrating succinate accumulation at 2.4 ppm. (C) Spectra acquired following 4 cycles of $^{177}$Lu-DOTATATE with no detectable succinate peak at 2.4 ppm. (D) Plasma metanephrine and methoxytyramine levels before and after treatment with $^{177}$Lu-DOTATATE. (E) Axial fused $^{18}$F-FDG PET/CT image and corresponding coronal PET projection showing the FDG-avid nodal metastases (SUV = 16.1, arrowed). (F) The same nodal metastases following treatment with [+Lu]-DOTATATE demonstrating reduced tracer uptake in keeping with the biochemical findings (SUV = 9.3).
A sequential $^1$H-MRS study was performed for patient 5 because of evidence of progressive disease on surveillance CT, despite treatment with a multikinase inhibitor, regorafenib. Serial $^1$H-MRS demonstrated a larger succinate peak (Fig 7.7E) compared with the first study (Fig 7.7D), and this correlated with the $^{18}$F-FDG avidity on PET/CT pre-treatment (Fig 7.7F) and 10 months post-treatment (Figure 7.7G), which demonstrated an increase in disease burden and avidity (standardized uptake value, 15.1 and 27.1, respectively).

Repeatability of $^1$H-MRS was evaluated in two patients by investigating different tumour deposits during the same study examination (patient 5) and the same tumour deposit twice during the same study examination (patient 1). The results for SCR were almost identical in these two patients, suggesting good test reproducibility (Table 7.3).

### Table 7.3: Characteristics of the two patients in whom $^1$H-MRS was repeated during the same examination to evaluate test reproducibility.

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumour site</th>
<th>Max tumour diameter on 1st scan</th>
<th>Maximum tumour diameter on 2nd scan</th>
<th>Succinate: choline ratio on 1st scan</th>
<th>Succinate: choline ratio on 2nd scan</th>
<th>Succinate detected on 1st scan</th>
<th>Succinate detected on 2nd scan</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Liver</td>
<td>2.6</td>
<td>2.6</td>
<td>0.74</td>
<td>0.72</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>5</td>
<td>Liver</td>
<td>2.5</td>
<td>2.3</td>
<td>0.15</td>
<td>0.17</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>
**Figure 7.7:** (A) T1-weighted MR image of a metastatic GIST to the liver (arrow) from case 5. (B) SDHB immunonegativity on SDHB immunohistochemistry performed on the wt GIST tumour from the same patient. (C) Axial fused 18F-FDG PET/CT image demonstrating an FDG-avid carotid body PGL after SDH deficiency was demonstrated on 1H-MRS. (D-E) Spectra acquired at 1H-MRS from the same case before and during treatment with a multi-kinase inhibitor. (F-G) Axial fused 18F-FDG PET/CT images and corresponding coronal PET projections illustrating the increase in disease burden and FDG avidity over time (SUV: 15.1 and 27.1) which correlates with the increase in the succinate peak demonstrated on 1H-MRS.
7.5 Discussion

This proof-of-principle study demonstrates that detection of a succinate peak and an increased SCR was specific for a variety of SDH-deficient tumour types. All six tumours with a positive succinate peak and elevated SCR were associated with a pathogenic germline SDHx variant (n = 4) or an SDHC epimutation (n = 2). In addition, the three patients with absent succinate peaks but adequate 1H-MRS demonstrated preservation of SDHB expression in the tumours analyzed. The findings of this study are complementary to a previous smaller study in which 1H-MRS was applied in nine patients with PGLs, and succinate peaks were detected in all five with pathogenic SDHx variants but not in the four patients without mutations(301). This study demonstrates for the first time to my knowledge that 1HMRS can also be used to determine the SDH status of GISTs and pituitary adenomas and that succinate peaks can be detected in SDH-deficient tumours with epigenetic inactivation of SDHC.

7.51: Identified translational applications of in vivo metabolomic analysis using 1H-MRS

Potential diagnostic applications of this new approach include: assessing the pathogenicity of patients with germ line SDHx variants of uncertain significance and potentially SDH-related tumours; investigating possible metastatic lesions (eg, in the liver) in patients with germline SDHx variants and primary SDH-deficient tumours; assessing patients with multiple primary tumors to determine if all are SDH deficient; identifying patients without detectable germline SDHx variants, who might benefit from specialist genetic investigations, such as SDHC promoter methylation status; and assessing SDH tumour status preoperatively, particularly for patients with possible wt GISTs, because standard adjuvant treatment with imatinib has proven to be less effective in patients with SDH-deficient disease (98).
This study demonstrates that $^1$H-MRS could be a valuable tool for the assessment of tumour response in the context of radionuclide and other therapies, because alterations in succinate levels were detected despite stable appearance of the tumour diameters. This important application of $^1$H-MRS could be expanded to include other tumours with specific metabolic defects, including fumarate hydratase–deficient tumors (66), $IDH1$-mutant tumors (298), and the recently identified malate dehydrogenase 2–deficient tumors (67).

7.52 Lessons learned regarding optimisation and limitations of in vivo metabolomic analysis using $^1$H-MRS in SDH deficient tumours

Notably, in this study choline signal was used as an internal control for viable tissue to discriminate technical failures from a negative finding. Interestingly, previous studies have only reported positive choline signals in those tumours that also had detectable succinate peaks (29).

To avoid issues of partial volume effects within smaller tumours, the voxel for MRS analysis was chosen to fully include tumour where possible. No statistically significant correlation between tumour size and SCR was detected, although there was a trend toward significance. This trend is the opposite of what would be expected if necrosis were artificially lowering the overall succinate levels in large tumours and therefore suggests that the method is measuring real differences in succinate, which are independent of tumour size. However, this study again suggests that a size threshold of >2 cm where possible should be applied, to improve the sensitivity of the test.

Important limitations of in vivo metabolomic analysis using $^1$H-MRS were also revealed by this study; for example, spectral quality was poor in close proximity to metal dental work, in areas adjacent to air spaces such as the lung, and in bone metastases and was susceptible to
motion artefact. In this study, the technical failure rate was 26%, which is similar to the failure rate reported in previous studies using $^1$H-MRS (300). Importantly, no patient cases were excluded from this prospective study, with the intention that this would inform the translation of this imaging modality into clinical practice. On the basis of the evidence from this exploratory study, it is advisable that tumours be selected for $^1$H-MRS analysis based on the following: ideally the largest tumour deposit but at least > 2 cm in size; tumours located close to bone or lung should be avoided; tumours with significant necrosis or haemorrhage should be avoided; superficial tumour deposits should be selected preferentially; and respiratory triggered acquisition should be used for tumours in the upper abdomen, such as hepatic metastases.

Although the use of $^1$H-MRS as a diagnostic tool is likely to be limited to specialist centres, the number of scan averages in our study during spectral acquisition was less than half those reported in a previous study (200 v 512) (301), without demonstrating a reduction in sensitivity. Using fewer scan averages reduces the acquisition time, making it more cost effective and convenient for the patient. This is a particularly important consideration if this imaging technique is to be considered for routine clinical practice or for sequential follow-up as part of a clinical trial.
7.5.3 Future applications of in vivo metabolomics analysis

There is increasing interest in understanding the metabolic adaptations that occur during tumorigenesis and how these might be exploited for novel therapeutic interventions as discussed in chapter 6. Increased production of lactate during aerobic glycolysis in most cancers, or the Warburg effect, is the best-known example of this. SDH-related cancers provide a paradigm for investigating tumour metabolism, because succinate is thought to act as an oncometabolite and to drive tumorigenesis. Therefore understanding the molecular mechanisms of SDH-related tumorigenesis provides a rationale for novel therapeutic interventions such as reversing the epigenetic abnormalities or exploiting metabolic vulnerabilities, similar to the recent discovery that tumoural 2-hydroxyglutarate, succinate and fumarate accumulation may increase responsiveness to olaparib, a poly (ADP-ribose) polymerase inhibitor (303)(304). The availability of sensitive non-invasive biomarkers would greatly facilitate precision medicine–based clinical trials in this field.

Furthermore although $^{18}$F FDG PET CT is a sensitive imaging tool, it lacks specificity and cannot differentiate individual metabolites. $^1$H-MRS is highly specific and allows in vivo detection of individual metabolites without the use of ionizing radiation; however, $^1$H-MRS is significantly less sensitive than PET, which could limit the detection of low levels of succinate, and it can be challenging to differentiate intracellular from extracellular metabolites. In the future, $^1$H-MRS may be complemented by other techniques, such as hyperpolarized carbon-13 MRS, which can increase MRS SNR by several orders of magnitude, allowing assessment of enzyme flux in vivo (305) or may indeed be used to complement existing functional imaging tests such as $^{18}$F-FDG PET-CT.
7.6 Conclusions

In conclusion, this study is the largest to date to my knowledge to evaluate $^1$H-MRS in patients with SDH-deficient tumours. It reveals that $^1$H-MRS has the potential to be used as a non-invasive biomarker in the precision management of SDH-deficient disease and could have a role as a biomarker of successful treatment response. Lessons learned from this study could be applied to other similar metabolically driven tumours.
Chapter 8

Investigating the role of *SDHC* epigenetic silencing in the pathogenesis of phaeochromocytoma/paraganglioma and GIST

**DECLARATION**: The findings reported in this study have been submitted for publication in Clinical Epigenetics.
Chapter 8: Investigating the role of SDHC epigenetic silencing in the pathogenesis of phaeochromocytoma/paraganglioma and GIST

8.1 Introduction

8.11 Epigenetic gene silencing in tumourigenesis

8.12 Carney’s triad

8.2 Aims

8.3 Methods

8.30 Clinical sample collection

8.31 Tumour microdissection and DNA extraction

8.32 Bisulfite modification

8.33 Polymerase chain reaction and pyrosequencing

8.34 RNA extraction

8.35 cDNA Synthesis

8.36 Expression Analysis with Quantitative RT PCR

8.37 Genetic sequencing

8.38 Analysis of TCGA tumour set

8.39 Statistical analysis

8.4 Results

8.41 Genotype and clinical phenotype of patient cohort

8.42 Methylation analysis by pyrosequencing

8.43 Analysis of SDHC gene expression

8.44 Tumour sequencing and additional function analysis for SDH deficiency in the hypermethylated cases

8.45 Whole genome sequencing analysis
8.46 Investigating *SDHC* hypermethylation in non PPGL and wt GIST tumour sets

8.5 Discussion

8.51 Phenotype of *SDHC* epimutant cases

8.52 Translating the diagnosis of an *SDHC* epimutation into clinical practice

8.6 Conclusion
8.1 Introduction

*Ex vivo* and *in vivo* targeted metabolomic analysis for succinate identified cases of SDH deficient tumours without a detectable germline or somatic *SDHx* variant. This prompted me to carry out a study to identify the role of *SDHC* epigenetic silencing in the pathogenesis of an unselected cohort of patients with PPGL and wt GIST.

8.11 Epigenetic gene silencing in tumourigenesis

Epigenetics is defined as a change in gene expression without a pathogenic alteration in DNA sequence and plays a fundamental role in human regulatory biology; including inactivation of the X chromosome and in embryonic development (306). DNA methylation is an epigenetic modification, which can affect gene expression and is the most studied epigenetic marker in cancer. DNA hypermethylation refers to the gain of a methyl group catalysed by the DNA methyl transferase enzymes at specific loci but with a preference for the CpG islands of a gene promoter (39). Hypermethylation in the promoter region of a gene can downregulate gene expression and initiate tumourigenesis if DNA hypermethylation affects the expression of tumour suppressor genes (307). Mechanisms by which DNA hypermethylation in the promoter region of a gene affects gene expression includes; i) inhibition of transcription factor binding and ii) recruitment of methyl-CpG binding domain proteins (308).

The advent of next generating sequencing coupled with pre-treatment by bisulfite modification has facilitated whole genome methylation analysis and the discovery of >100 genes and gene products affected by hypermethylation in cancer (309). An example of gene specific epigenetic silencing in cancer predisposition syndromes includes; Lynch syndrome caused by epigenetic silencing of the mismatch repair genes *MLH1* and *MSH2* (310) and *BRAC1* and *BRAC2* epigenetic repression in breast and ovarian cancer (41).
One of the major benefits of early identification of epigenetic modifications in cancer is the potential for reversibility and consequently these epigenetic modifications are optimal therapeutic targets. At present, two DNA methyltransferase (DNMT) inhibitors (5-azacytidine and 5-aza-2'-deoxycytidine) are approved for use in myelodysplastic syndrome and leukaemia (311). These agents have demonstrated poor efficacy and considerable toxicity in the treatment of solid tumours and recent studies have focused on the development of new second generation DNMT inhibitors with better tolerability profiles (312).

### 8.13 Carney’s triad

Carney’s triad (CT) was first coined by Dr J. Adian Carney in 1977, as a new specific form of multiple endocrine neoplasia, consisting of the triad of i) extra adrenal paraganglioma, ii) gastrointestinal stromal tumour (GIST) and iii) a pulmonary chondroma (PCHO) (313). This syndrome shows clinical overlap with Carney’s Stratakis syndrome (CSS) described in 2002, referring to the dyad of GIST and PPGL caused by a pathogenic germline variant in one of the four SDH subunit genes (SDHA/SDHB/SDHC/SDHD) (101). Initially CT was attributed to a large genomic deletion on 1q encompassing the SDHC gene (314), however this finding was not confirmed by subsequent studies (302)(102)(315). Recently it has been suggested that CT is not caused by a germline genetic variant but rather an aberrant methylation pattern in the promoter region of the SDHC gene at a tumour level (302)(102)(315). Detection of hypermethylation at the promoter region of the SDHC gene in the tumour has correlated with reduced expression of SDHC mRNA and reduced expression of SDHB and SDHC protein expression, indicating that epigenetic inactivation of the SDHC gene can lead to SDH enzyme deficiency and initiate tumorigenesis (102)(315). Interestingly, the methylation status of the promoter region of the other SDHx gene subunits has been investigated, with no evidence of
hypermethylation or reduced mRNA expression of SDHA, SDHB or SDHD genes (302)(315). Furthermore, studies to date have not identified this locus specific methylation pattern in over 2000 control samples, including tumour samples with a presumed hypermethylator epigenotype (IDH1 mutant gliomas) (102) or in breast cancer tumour samples or cell lines (316). One hypothesis for this focal hyper-methylation is that a structural genetic variation upstream from the SDHC gene promoter region may be accounting for secondary aberrant methylation, but again a previous study failed to identify a genetic sequence anomaly on a 130-kbp window of whole genome sequencing data around the SDHC gene locus of 15 SDHC epi-mutant GIST tumour samples (102).

It also remains unclear as to whether the epigenetic silencing of the SDHC gene is a constitutional, postzygotic, or somatic phenomenon. One recent study favoured a postzygotic mechanism (102). Intriguingly, this epimutation has been reported more commonly in females than males and is most commonly associated with the development of wtGIST (102)(315)(317)(98)(318). However, Carney originally reported a significant delay in the development of all three tumour types (8 years), therefore the higher proportion of SDHC epimutant GIST tumours reported in the literature may represent a forme fruste of Carney’s triad.

Ex vivo and in vivo metabolomics analysis performed by our group and others (260)(319), has demonstrated that pathological succinate accumulation occurs in tumours with SDHC epigenetic silencing and therefore the mechanisms by which the oncometabolite succinate drives tumourigenesis should be similar for the SDHC epimutant and SDH mutant tumours. The key difference is that the SDHC epimutation like other epigenetic aberrations is potentially reversible. This potential for reversing an oncogenic driver is particularly relevant at present with an active phase two study investigating the role of a second generation DNA
methyltransferase inhibitor (Guadecitabine, SGI-110) in wt GIST with both SDHx germline variants and SDHC epimutations (ClinicalTrials.gov Identifier: NCT03165721).

Diagnosing an SDHC epimutation is important for a number of reasons; i) firstly it could inform management and surveillance approaches for those patients diagnosed with an SDHC epimutation, ii) it can help alleviate anxiety within a family about a potential pathogenic germline gene variant and iii) it may enable personalized therapeutic strategies for patients with malignant disease.

There are currently no published methods for SDHC protein immunohistochemistry and the indications for testing and a suitable methodology for a clinical service laboratory have not yet been defined. Therefore investigating and validating a novel pyrosequencing-based assay for SDHC promoter methylation and suggesting a pathway for implementing testing in clinical diagnostics will be crucial in order to improve diagnostic yield of this rare but important epigenetic aberration.
Figure 8.1: This figure illustrates the tumour components of Carney’s triad highlighted by a red arrow in each image. Figure A shows a wt GIST, figure B demonstrates an abdominal PGL and figure C shows a large pulmonary chondroma.

8.2 Aims

1. To investigate indications and diagnostic methodologies and pathways for the detection of an SDHC epimutation in a service setting
8.3 Methods

8.30 Clinical sample collection and study design

Cases were ascertained from the Neuroendocrine Tumour and the National Pediatric and Adult wild type GIST (PAWS GIST UK) clinics at Cambridge University Hospital Foundation Trust and the West Midlands and East Anglian Regional Genetic Services. All cases of identified PPGL and wild type GIST, for whom FFPE tumour blocks were available, were considered for inclusion in the study.

FFPE tumour blocks were collected from 45 patients with either PPGL or wt GIST. All participants gave written informed consent and the study was approved by South Birmingham Research Ethics Committee (REC reference number: 5175). Thirteen cases were excluded from further study because insufficient DNA (concentration <1000ng) was extracted from the tumour block leaving 32 cases (15 wt GIST and 17 PPGL) for further analysis. For each case studied, DNA was extracted from FFPE tumour tissue and adjacent normal tissue (31/32 cases) and blood (21/32 cases). cDNA was extracted from FFPE tumour tissue and adjacent normal FFPE tissue. Data on the clinical phenotype, family history and germline molecular test results was collated from patient records. Methylation analysis was performed on DNA extracted from FFPE tumour and matched normal tissue/blood. SDHC expression analysis was performed on RNA extracted from FFPE tumour and matched normal tissue. SDHB immunohistochemistry was performed on all tumours and tumour samples with evidence of SDHB preservation on SDHB IH were included in SDHC promoter methylation analysis in order to confirm if SDHB IH was a sensitive triaging test for the diagnosis of an SDHC epimutation. Sequencing of tumour DNA was performed to identify somatic SDHx variants.
A further 17 *IDHI* mutant glioma samples (anonymised tumour DNA consented patients provided by Professor Colin Watts) were included in the study with an assumed hypermethylated epigenome, to further test the hypothesis that the *SDHC* epimutation is unique to specific tumour types and a distinct epigenetic entity.

**8.31 Tumour microdissection and DNA extraction**

See methods section, chapter 2

Methods 8.32-8.37 were performed by Dr Rogier ten Hoopen. Experimental design and methodologies were independently chosen. Experimental errors were reviewed and solved independently after discussion with Dr ten Hoopen and all experimental data was independently analyzed.

**8.32 Bisulfite modification**

250-1000 ng of DNA was bisulfite modified with the Zymo Research EZ DNA Methylation kit (D5001) according to the manufacturer’s instructions.

**8.33 Polymerase chain reaction and pyrosequencing**

A PCR amplicon in the CpG27 region island located in the SDHC promoter gene of the SDHC gene was amplified on bisulfite converted DNA with primer Fwd-SDHC (GAAAATATTAGTAAATTAGTTAG) and 187.5nM of biotinylated reverse primer RevBio-SDHC: (ACTAAAATCACCTCAACAAAAC) with the Qiagen PyroMark
kit (Qiagen 978703). The PCR conditions were 7 min at 95°C, followed by 20 sec at 95°C, 30 sec at 53°C, and 20 sec at 72°C for 42 cycles, and an end incubation at 72°C for 5 min. The resulting PCR amplicon was quality assessed on a 2% agarose gel before running 10ul of the sample on the Qiagen Q24 pyrosequencer as per manufacturer’s instructions. Fully methylated and unmethylated human control DNA that had been treated with bisulfite were used as controls on each pyrosequencing run (DNA; Qiagen).

8.34 RNA extraction

See methods section, chapter 2.

8.35 cDNA Synthesis

See methods section, chapter 2.

8.36 Expression Analysis with Quantitative RT PCR

Relative expression analysis was performed with a predesigned Taqman Gene Expression assay for $SDHC$ (SDHC: Hs01698067_s1) (Applied Biosystems, Hs01698067_s1, FAM) and using GAPDH as a reference gene (Applied Biosystems, Hs02758991_g1, FAM). Each sample was run in triplicates on Applied Biosystems Life Technologies QuantStudio 6Flex optical thermal cycler. The PCR conditions were 10 min at 95°C primary activation/denaturation step, followed by 45 cycles of a 15sec 95°C denaturation and a 1min 60°C annealing/elongation step at which time the fluorescence of the Taqman probe was measured.
8.37 Targeted genetic sequencing

i) See methods section, chapter 2.

iii) Whole genome sequencing

Whole genome sequencing (WGS) was performed on germline DNA from three cases as part of the Rare Disease BioResource BRIDGE project. Sequencing data from two of the three patients was included in a recent publication from this group (200). Data was filtered to exclude data outside of regions of interest that included the SDHC promoter region and genes involved in methylation maintenance and regulation including; TET1, TET2, TET3, DNMT3 and DNMT3B.

The variants were annotated with variant effect predictor and filtered on i) minor allele frequency of <0.1 or absent in 1000 genome project (www.internationalgenome.org) and UK10K (https://www.uk10k.org), ii) consequence including; truncating, missense, splice site and in frame deletion and insertion variants and iii) quality including; a read depth of >10 and variant allele frequency of >0.3. All filtered variants were then individually interrogated by myself and assigned pathogenicity based on American College of Medical Genetics and Genomics (ACMG) criteria. A comparison of variant frequencies in our samples compared to a control group with low neoplastic risk within the bio resource project (NIHR rare disease controls, n=4053), was also performed and calculated using a Fishers exact test and corrected for a false discovery rate using the Benjamani Hochberg method. Finally, cases were evaluated for structural variants (SV) including copy number variation, using the SV calling tools; Canvas and/or Manta (320)(321).
8.38 Analysis of TCGA tumour set

The c-bioportal (http://www.cbioportal.org) dataset was interrogated for tumours with SDHC expression below the median expression level for the given tumour type and without a sequence mutation. I identified thirty tumours and 450k infimum methylation array data was available for 25/30 tumours from the cancer genome atlas (TCGA) (https://cancergenome.nih.gov). Open access data was downloaded for the 25 tumours and analysed for evidence of SDHC promoter methylation based on the β value across SDHC promoter CpG targets mapping to the TSS-proximal CGI (cg00576014, cg01931502, cg11221228, cg12036621, cg17496230, cg11744295, cg08716396, cg15152945, cg02656741, cg02620307, cg09686639, cg12036621, cg01931502).

8.39 Statistical analysis

R studio (1.1.447) was employed for analysis of differential SDHC mRNA expression and for methylation analysis and visualisation. Statistical analysis was performed using MedCalc (version 18.2.1). A mean and standard deviation was calculated for all continuous variables. An unpaired student t-test was employed to investigate differences between groups and an analysis of variation (ANOVA) was used to determine variation across experimental groups.

ROC curve analysis was performed to determine the optimal mean methylation cut off to differentiate SDHC epimutant cases from non epimutant cases. A sample size of 40 was required (10 positive cases and 31 negative cases) to ensure a minimum type 1 and type 2 error of 0.2. Therefore to achieve this sample size data from this study was combined with the data published by Haller et al (315) (including four cases with CT and five negative control samples) for ROC curve analysis.
8.4 Results

8.41 Genotype and clinical phenotype of patient cohort

A total of 32 patients were included in the final study. The mean age of participants was 36.6 years (range 15-71, SD 18.8). Thirteen participants were male, 19 female. The phenotypes included 17 (53%) cases of PPGL, 15 (47%) patients with wt GIST and nine patients (28%) including 7 GIST and 2 PPGL cases, had metastatic disease at the time of this study. Five patients had a clinical history of multiple tumours: three patients with a wt GIST and PGL, one patient with a carotid body PGL and an abdominal PGL and one patient with a wt GIST, esophageal leiomyoma and a pulmonary chondroma (Table 8.1).

The fifteen cases of wtGIST included 10 cases of dSDH-wtGIST and 5 cases of SDH preserved wtGIST, as defined by loss or preservation respectively of SDHB protein expression on immunohistochemistry. The seventeen PPGL cases included 13 SDH preserved PPGL, 3 dSDH PPGL and 1 PPGL with an equivocal SDHB result (case # 026) (Table 8.1).

A likely pathogenic or pathogenic germline variant was identified in 12/32 patients (37.5%; 6/15 GIST and 6/17 PPGL) (Table 8.1). A positive family history was identified in case #032 with a wt GIST, whose daughter had a diagnosis of a glomus PGL and a family history of PPGL was noted for case #026 with VHL.
Table 8.1: Clinical and molecular profile of patients with wtGIST

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age</th>
<th>Sex</th>
<th>Pathogenic germline variant</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>23</td>
<td>F</td>
<td>Negative</td>
<td>Metastatic wt GIST</td>
</tr>
<tr>
<td>002</td>
<td>15</td>
<td>F</td>
<td>Negative</td>
<td>Metastatic wt GIST</td>
</tr>
<tr>
<td>003</td>
<td>21</td>
<td>F</td>
<td>Negative</td>
<td>Metastatic wt GIST</td>
</tr>
<tr>
<td>004</td>
<td>24</td>
<td>F</td>
<td><em>SDHC</em> c.380A&gt;G, p.His127Arg</td>
<td>wt GIST+ Oesophageal leiomyoma + Pulmonary chondroma</td>
</tr>
<tr>
<td>005</td>
<td>22</td>
<td>F</td>
<td><em>SDHB</em> c.380G&gt;T p.Ille127Ser</td>
<td>Abdominal PGL</td>
</tr>
<tr>
<td>006</td>
<td>27</td>
<td>M</td>
<td><em>SDHB</em> c.302G&gt;A p.Cys101Tyr</td>
<td>Abdominal PGL+ Carotid PGL</td>
</tr>
<tr>
<td>007</td>
<td>15</td>
<td>M</td>
<td>Negative</td>
<td>Abdominal PGL</td>
</tr>
<tr>
<td>008</td>
<td>21</td>
<td>M</td>
<td>Negative</td>
<td>PC</td>
</tr>
<tr>
<td>009</td>
<td>40</td>
<td>F</td>
<td>Negative</td>
<td>Metastatic PC</td>
</tr>
<tr>
<td>010</td>
<td>38</td>
<td>F</td>
<td><em>NF1</em> c.1318C&gt;T p.Arg440Ter</td>
<td>PC</td>
</tr>
<tr>
<td>011</td>
<td>78</td>
<td>F</td>
<td>Negative</td>
<td>PC</td>
</tr>
<tr>
<td>012</td>
<td>38</td>
<td>F</td>
<td><em>RET</em> c.1900T&gt;A p.Cys634Ser</td>
<td>PC</td>
</tr>
<tr>
<td>013</td>
<td>30</td>
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<td>M</td>
<td>Negative</td>
<td>PC</td>
</tr>
<tr>
<td>015</td>
<td>37</td>
<td>F</td>
<td><em>RET</em> c.1900T&gt;A p.Cys634Ser</td>
<td>PC</td>
</tr>
<tr>
<td>016</td>
<td>52</td>
<td>M</td>
<td>Negative</td>
<td>PC</td>
</tr>
<tr>
<td>017</td>
<td>78</td>
<td>M</td>
<td>Negative</td>
<td>PC</td>
</tr>
<tr>
<td>018</td>
<td>45</td>
<td>M</td>
<td>Negative</td>
<td>Abdominal PGL</td>
</tr>
<tr>
<td>019</td>
<td>16</td>
<td>F</td>
<td><em>SDHA</em> c.91C&gt;T p.Arg31Ter</td>
<td>Metastatic wt GIST</td>
</tr>
<tr>
<td>020</td>
<td>37</td>
<td>M</td>
<td><em>SDHB</em> c.137G&gt;A p.(Arg46Gln)</td>
<td>Metastatic wt GIST+ Carotid PGL</td>
</tr>
<tr>
<td>021</td>
<td>21</td>
<td>F</td>
<td><em>SDHD</em> c.34G&gt;A (p.Gly12Ser)</td>
<td>Metastatic wt GIST+ Thoracic PGL</td>
</tr>
<tr>
<td>022</td>
<td>27</td>
<td>F</td>
<td><em>SDHC</em> c.148C&gt;T (p.Arg50Cys)</td>
<td>wt GIST+ Abdominal PGL</td>
</tr>
<tr>
<td>023</td>
<td>29</td>
<td>F</td>
<td>Negative</td>
<td>wt GIST</td>
</tr>
<tr>
<td>024</td>
<td>36</td>
<td>F</td>
<td><em>NF1</em> c.4421delG p.(Tyr794Ter)</td>
<td>wt GIST</td>
</tr>
<tr>
<td>025</td>
<td>72</td>
<td>M</td>
<td>Negative</td>
<td>PC</td>
</tr>
<tr>
<td>026</td>
<td>25</td>
<td>F</td>
<td><em>VHL</em> c.499C&gt;G (p.Arg167Gly)</td>
<td>PC</td>
</tr>
<tr>
<td>027</td>
<td>22</td>
<td>F</td>
<td>Negative</td>
<td>wt GIST</td>
</tr>
<tr>
<td>028</td>
<td>24</td>
<td>F</td>
<td><em>SDHA</em> c.1909-2A&gt;G</td>
<td>Metastatic wt GIST</td>
</tr>
<tr>
<td>029</td>
<td>27</td>
<td>F</td>
<td>Negative</td>
<td>Metastatic PC</td>
</tr>
<tr>
<td>030</td>
<td>30</td>
<td>M</td>
<td>Negative</td>
<td>wt GIST</td>
</tr>
<tr>
<td>031</td>
<td>57</td>
<td>M</td>
<td>Negative</td>
<td>wt GIST</td>
</tr>
<tr>
<td>032</td>
<td>67</td>
<td>M</td>
<td><em>SDHD</em> c.296delT, p.Leu99Profs*36</td>
<td>wt GIST</td>
</tr>
</tbody>
</table>
8.42 Methylation analysis of tumour DNA by pyrosequencing

The % methylation at each of the 12 CpG’s in CpG island 27(CpG27) in the promoter region of SDHC was tested (Figure 8.2). The percentage methylation ranged between 1% and 73% but was highly correlated within an individual tumour sample, with no significant variability detected across individual CpG’s (p=0.08) (see Figure 8.3A+B). A mean % methylation of 2.2% (SD 1.98) across 12 CpG’s, was detected in all but 6 (18.7%) tumour samples. The mean methylation in these 6 tumours (cases; #001, #002, #003, #004, #021, #022) was 50.8% (SD 16.4) (Figure 8.3B)

Comparison of the 6 tumours with evidence of SDHC hypermethylation to those with low methylation revealed an association with wtGIST (6/15 versus 0/17 PPGL; P=0.005), female (6/19 versus 0/13 males; P=0.02); metastatic disease (5/6 versus 4/26 (P=0.03), younger age at diagnosis (mean age 24 years, range 15-27 years versus mean age 39.2 years, range 16-78 years) (p=0.0002)) and multiple primary tumours (3/6 versus 2/26, P=0.03) but not the presence of a germline pathogenic SDHx variant (2/6 versus 3/26 P=0.2) (Table 8.1). No case of PPGL with SDHC hypermethylation was identified in this study.

Pyrosequencing of blood DNA was performed on 22/32 (69%) and matched normal tissue for 31/32 (97%). No evidence of SDHC promoter hypermethylation was detected (all CpGs <10% in all samples) including in the 6 samples with tumour SDHC hypermethylation (one of whom (case #004) has multiple tumours). No statistically significant difference was identified between the mean % methylation of blood DNA or adjacent normal tissue for those cases identified as having tumour hypermethylation compared with those cases without tumour methylation (p=0.6) (Figure 8.4C). A significant difference was noted for the % methylation in the tumour compared to the adjacent normal tissue for the 5/6 hypermethylated cases (p=0.003) (Figure 8.4A). Adjacent normal tissue was not available
for one of the hypermethylated cases (#001). Finally, ROC curve analysis confirmed that a methylation of >8.5% separated the cases with an identified epimutation and silencing of SDHC from those without (AUC 1.0, p=<0.0001).

8.43 Analysis of SDHC gene expression

To investigate whether SDHC promoter methylation was associated with transcriptional silencing, analysis of SDHC mRNA in both tumour tissue and adjacent normal tissue was performed in 31/32 cases. In 5/5 tumour samples with SDHC hypermethylation the mean fold difference was -6.41 (SD 5.4) (Figure 8.4B) compared to 1.41 (SD 4.41) in 26 tumours without SDHC hypermethylation (P=0.0002) (Figure 8.5). Though most tumours without SDHC hypermethylation showed higher levels of SDHC expression in tumours than adjacent normal tissue (23/26), three tumours showed reduced expression, the lowest of which (#0026) had a -15-fold difference. This phaeochromocytoma was associated with a known pathogenic germline VHL variant (c.499C>G, p.Arg167Gly) and the phaeochromocytoma showed loss of SDHB protein expression on immunohistochemistry but no evidence of a somatic SDHx mutation.
Figure 8.2: Figure A demonstrates an adaption of a UCSC (University of California, Santa Cruz) genome browser display of SDHC genomic position (hg38 coordinates) and CpG islands 17 and 27. CpG island 27 (CpG27) was investigated in this study and the utilised primers are displayed. Figure B shows the pyrosequencing data from case #001, showing evidence of hypermethylation across the 12 analysed CpG’s
Figure 8.3: Figure A illustrates the distribution of methylation across the 12 individual CpG’s for the six cases demonstrated to have SDHC promoter methylation (epimutant cases), and the wt GIST, glioma and PPGL cases with no SDHC epimutation. Figure B demonstrates the methylation levels across the 12 individual CpG’s for the six epimutated cases (#001, #002, #003, #004, ##021, #022).
Figure 8.4: Figure A shows a significant difference in the mean % methylation of the SDHC promoter locus in the tumour tissue of the 6 cases with an identified SDHC epimutation compared to the mean methylation in the adjacent normal tissue. Figure B shows reduced SDHC expression in the tumour versus normal tissue of 5/6 cases with an identified SDHC epimutation. Figure C demonstrates the mean % methylation of the SDHC promoter locus across 12 CpG’s in the blood DNA and normal tissue of cases with and without an identified SDHC epimutation.
**Figure 8.5:** This histogram demonstrates the fold difference of *SDHC* mRNA expression (corrected for GAPDH) in tumour compared to adjacent normal tissue. Those bars represented in grey show a negative fold difference in tumour compared to normal tissue and black bars represent a positive fold difference in tumour compared to normal tissue.
8.44 Tumour sequencing and additional functional analysis for SDH deficiency in the hypermethylated cases

Tumour sequencing was performed on 4/6 (#001, #002, #003, #004) cases with evidence of *SDHC* hypermethylation and no somatic *SDHx* variants were detected. SDHB immunohistochemistry was performed on all tumours and loss of SDHB expression was confirmed in all 6 cases with *SDHC* hypermethylation (Figure 8.6 B+D).

8.45 Whole genome sequencing analysis

WGS data was analysed for three cases with tumour *SDHC* hypermethylation (cases; #002, #021 and #022). No candidate pathogenic structural or single nucleotide variants were identified in these three cases in the *SDHC* locus (between 161314257-161375340) containing the SDHC promoter, exons and 3'UTR). In the absence of an in cis genetic cause, additional analysis for potential pathogenic variants in genes implicated in genome methylation (*TET1, TET2, TET3, DNMT3B, DNMT3A, DNMT1*), was performed and 10/965 filtered variants (in test and control samples) were detected in 3 genes (Table 8.2). A comparison of the identified variant frequencies in the three SDHC hypermethylation samples compared to 4053 control genomes with low neoplastic risk (from the NIHR Rare Diseases BioResource BRIDGE project) did not yield any statistically significant findings (Benjamani Hochberg correction for a false discovery rate of p values was applied and based on 965 tested hypotheses).

None of the variants identified in the SDHC methylation cases were considered to be pathogenic by ACMG criteria, although a missense variant of uncertain significance in *TET2* (p.Ile1762Val) was identified in all three cases with *SDHC* promoter hypermethylation, this variant was absent from 1000 genomes and UK10K databases, was identified in 1876/4053
controls (Table 8.2) and has a minor allele frequency (MAF) of 0.2 on EXAC (http://exac.broadinstitute.org).

8.46 Investigating SDHC hypermethylation in non PPGL and wt GIST tumour sets

To investigate further the apparent specificity of SDHC epimutations to wtGIST, I explored whether SDHC epimutations might occur in non wtGIST tumours with (a) DNA hypermethylation or (b) low SDHC expression. Firstly 17 IDH1 mutant glioma tumours (IDH1 mutant glioma tumours have previously been associated with a global hypermethylation phenotype due to inhibition of alpha ketoglutarate dependent demethylation enzymes) were analysed for SDHC epimutations. All 17 samples had the same somatic IDH1 mutation (c.395G>A, p.Arg132His). The mean SDHC promoter methylation was low at 2% (SD 1.28, range 1-4%) (Figure 8.3B). Secondly, from non-wtGIST tumours with SDHC gene expression data and sequencing data from cancer genome analysis studies we identified 25 tumour samples with very low SDHC transcript levels and no SDHC mutation (Table 8.3). Methylation array (illumine 450k) data for these 25 tumours was accessed and beta values for 13 SDHC promoter probes inspected. None of the tumours showed evidence of SDHC promoter methylation (Table 8.3).
Table 8.2: Table of variants identified in genes associated with regulation of methylation on whole genome sequencing analysis

<table>
<thead>
<tr>
<th>RS ID</th>
<th>Gene</th>
<th>Protein change</th>
<th>EXAC</th>
<th>Number of cases</th>
<th>Control</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs61741171</td>
<td>TET3</td>
<td>p.(Pro294Ser)</td>
<td>0.04</td>
<td>1</td>
<td>441</td>
<td>Uncertain significance</td>
</tr>
<tr>
<td>rs6843141</td>
<td>TET2</td>
<td>(p.Val218Met)</td>
<td>0.05</td>
<td>1</td>
<td>133</td>
<td>Uncertain significance</td>
</tr>
<tr>
<td>rs17253672</td>
<td>TET2</td>
<td>(p.Pro363Leu)</td>
<td>0.04</td>
<td>1</td>
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<td>rs34402524</td>
<td>TET2</td>
<td>(p.Leu1721Trp)</td>
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<td>1</td>
<td>881</td>
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</tr>
<tr>
<td>rs2454206</td>
<td>TET2</td>
<td>(p.Ile1762Val)</td>
<td>0.3</td>
<td>3</td>
<td>1876</td>
<td>Uncertain significance</td>
</tr>
<tr>
<td>rs10823229</td>
<td>TET1</td>
<td>(p.Asp162Gly)</td>
<td>0.3</td>
<td>1</td>
<td>1872</td>
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</tr>
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<td>rs12773594</td>
<td>TET1</td>
<td>(p.Ser193Thr)</td>
<td>0.1</td>
<td>1</td>
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<td>rs12221107</td>
<td>TET1</td>
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<td>1</td>
<td>523</td>
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<tr>
<td>rs16925541</td>
<td>TET1</td>
<td>(p.Asn1018Ser)</td>
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<td>1</td>
<td>488</td>
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<td>rs199882600</td>
<td>TET1</td>
<td>(p.Val2128Ile)</td>
<td>.0004</td>
<td>1</td>
<td>2</td>
<td>Uncertain significance</td>
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Table 8.3: TCGA tumour set with differential SDHC expression analysed for SDHC promoter hypermethylation

<table>
<thead>
<tr>
<th>TCGA ID</th>
<th>Tumour type</th>
<th>Mean β-value across SDHC promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCGA-5P-A9K2</td>
<td>Papillary RCC</td>
<td>0.06</td>
</tr>
<tr>
<td>TCGA-G7-A8LB</td>
<td>Papillary RCC</td>
<td>0.04</td>
</tr>
<tr>
<td>TCGA-B1-A47N</td>
<td>Papillary RCC</td>
<td>0.05</td>
</tr>
<tr>
<td>TCGA-P4-A5EA</td>
<td>Papillary RCC</td>
<td>0.13</td>
</tr>
<tr>
<td>TCGA-P4-A5ED</td>
<td>Papillary RCC</td>
<td>0.05</td>
</tr>
<tr>
<td>TCGA-DX-A2JO</td>
<td>Sarcoma</td>
<td>0.06</td>
</tr>
<tr>
<td>TCGA-DX-AB2V</td>
<td>Sarcoma</td>
<td>0.06</td>
</tr>
<tr>
<td>TCGA-DX-A48N</td>
<td>Sarcoma</td>
<td>0.05</td>
</tr>
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<td>TCGA-QR-A708</td>
<td>PPGL</td>
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</tr>
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<td>TCGA-KL-8327</td>
<td>Chromophobe RCC</td>
<td>0.06</td>
</tr>
<tr>
<td>TCGA-46-6026</td>
<td>Squamous cell Lung carcinoma</td>
<td>0.06</td>
</tr>
<tr>
<td>TCGA-BO-4811</td>
<td>Clear cell RCC</td>
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</tr>
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<td>TCGA-CF-A3MF</td>
<td>Bladder Cancer</td>
<td>0.05</td>
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<td>TCGA-VD-AA80</td>
<td>Uveal melanoma</td>
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</tr>
<tr>
<td>TCGA-OR-A5JX</td>
<td>Adrenocortical carcinoma</td>
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<tr>
<td>TCGA-BS-A0V7</td>
<td>Uterine Cancer</td>
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<td>TCGA-ZM-AA05</td>
<td>Testicular germ cell tumour</td>
<td>0.06</td>
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<tr>
<td>TCGA-ZM-AA06</td>
<td>Testicular germ cell tumour</td>
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<tr>
<td>TCGA-HC-7752</td>
<td>Prostate Cancer</td>
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<tr>
<td>TCGA-KK-A59V</td>
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<td>TCGA-XU-AAxz</td>
<td>Thymoma</td>
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<tr>
<td>TCGA-EM-A2CR</td>
<td>Thyroid Cancer</td>
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<td>TCGA-EM-A4FR</td>
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<td>TCGA-AB-2952</td>
<td>AML</td>
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8.5 Discussion

8.51: Phenotype of SDHC epimutant cases

SDHC promoter region methylation was identified in 6/15 wtGIST but none of 17 PPGL. All SDHC hypermethylation cases were female and were significantly younger than patients without an SDHC epimutation. Previously, SDHC promoter methylation has been reported in 27 cases (14 cases of wt GIST alone, two cases of PGL alone and 11 cases with a multi-tumour syndrome consisting of wt GIST and or PGL/PCHO) (102) (315) (322) (318) (265). Combining these results with previously published series, the association with wtGIST (alone or as the presenting feature of a multi tumour syndrome), females and young age at diagnosis is maintained, with rare reports of isolated PGL.

In two of our cases, SDHC promoter methylation was detected in the presence of a pathogenic germline pathogenic SDHC variant. This would be consistent (though not proven) with a two hit model of tumourigenesis in which SDHC hypermethylation resulted in silencing of the wild-type allele in the tumour. Two of the cases with a germline SDHC variant had multiple tumours including case #004 (wtGIST, PCHO and esophageal leiomyoma, and SDHC: c.380A>G, p.His127Arg). Although it was previously suggested that PCHO occurred exclusively in CT (a non-inherited disorder), this study and others (98) (323) have demonstrated that the triad of wtGIST, PGL and PCHO can occur in association with a germline SDHx variants and highlights the overlapping features of CT and CSS (313) (323) (324).

Though the phenotype associated with germline SDHx variants has evolved since the first reported associations with HNPGL and PPGL (59) (60) and it is now recognized that SDHx mutations can rarely predispose to non-clear cell RCC (325) and pituitary tumours (111) I
did not (from interrogation of TCGA, literature and original data) find evidence that \textit{SDHC} promoter methylation occurs outside of wtGIST and, occasionally, PGL.

Four cases of tumour \textit{SDHC} promoter methylation with no detectable germline or somatic \textit{SDHC} variants were identified. Furthermore there was no evidence of a germline \textit{SDHC} epimutation. In such cases the \textit{SDHC} promoter hypermethylation might be a somatic event as occurs in many types of cancer and multiple tumour suppressor genes (326). In the case of the mismatch repair gene \textit{MLH1}, somatic \textit{MLH1} promoter methylation is relatively common in older individuals with colorectal cancer with microsatellite instability but there are rare cases of patients with a constitutional \textit{MLH1} epimutation who present at a younger age (327). In contrast to \textit{MLH1}, there has been no evidence to date to a support an overt constitutional \textit{SDHC} epimutation, although some studies have suggested mosaic constitutional \textit{SDHC} promoter hypermethylation (102). Furthermore although patients with wtGIST and no evidence of a germline variant or epimutation have a young age at diagnosis (mean age 18 years), this is not significantly younger than those with a pathogenic germline \textit{SDHC} variant (mean age 26 years) (p=0.07). Also some patients with somatic \textit{SDHC} promoter methylation and no apparent germline \textit{SDHC} variants/epimutation have developed multiple primary tumours (n=1 in our study and 9 cases in the literature). These observations raise the possibility that there might be an undetected \emph{in cis or in trans} genetic variant in these patients that predisposes to somatic \textit{SDHC} promoter hypemethylation. However, in the three cases analysed by whole genome sequencing, no clear candidate genetic causes were identified.
ii) Translating the diagnosis of an *SDHC* epimutation into clinical practice

A primary aim of this study was to develop a clinical protocol for appropriate testing for *SDHC* promoter methylation. A pyrosequencing method was employed because this has been used in our local clinical diagnostic services for other types of testing (*MGMT* promoter methylation analysis in gliomas). The method adopted worked well on DNA extracted from FFPE tumour blocks (an important consideration as fresh frozen tumour is rarely available).

This study determined that the methylation status of 12 CpG’s in the promoter region of the *SDHC* gene could be accurately assessed on FFPE derived DNA and that hypermethylation of the *SDHC* promoter correlated with reduced *SDHC* mRNA expression in the tumour. ROC curve analysis demonstrated that a methylation of >8.5% separated the cases with a hypermethylation from those without (type 1 and type 2 error 0.20) and I propose a diagnostic cut-off of >10% for the diagnosis of hypermethylation at this locus. Whenever possible, cases with hypermethylation (mean methylation >10%) should then be analysed by RT-PCR of both tumour and adjacent normal tissue to confirm silencing of *SDHC* in the tumour tissue.

Given that SDHB immunohistochemistry is a relatively accessible and sensitive test, this should be considered as the first triaging test for the detection of SDH deficiency in PPGL and wt GIST and germline genetic sequencing should be simultaneously initiated (324). If germline genetic testing is negative and SDHB IH suggests loss of SDHB protein expression, the first step for PPGL should be somatic sequencing (212) to investigate for somatic *SDHx* or *VHL* variants, which can account for loss of SDHB protein expression (7) (Figure 8.6A).

However, as *SDHC* epimutations are more commonly implicated in wt GIST, I would recommend *SDHC* promoter methylation analysis as the next step after germline genetic testing for wt GIST (Figure 8.6B). If an *SDHC* epimutation is diagnosed, somatic tumour
sequencing should be performed to identify a co-existing somatic $SDHx$ variant, which may affect the efficacy of any potential de-methylating therapy (Figure 8.6).

Importantly, a number of potential limitations in the diagnosis of $SDHC$ methylation using pyrosequencing methods on FFPE tumour tissue, were encountered over the course of this study. Identification of these pitfalls has prompted the following practical recommendations; i) using a minimum input of 50ng of bisulfite converted DNA for the PCR and ii) a minimum volume of 10 microlitre for pyrosequencing can minimize the risk of false elevations in methylation, iii) fully methylated and unmethylated human control DNA, treated with bisulfite should be used as external controls on each pyrosequencing run and iv) the use of matched normal tissue is useful as an internal control to account for any false elevation in methylation which may have been caused by the long term paraffin storage. Finally data from this primary analysis as well as our interrogation of TCGA data, would suggest that detection of reduced $SDHC$ expression is not a sensitive test for the diagnosis of $SDHC$ hypermethylation and detection of an $SDHC$ epimutation is more accurately assessed by a combination of methylation followed by expression analysis.

Improving the accessibility of clinical testing for establishing tumour and constitutional $SDHC$ promoter methylation status is crucial as it makes an important contribution to SDH deficient wt GIST (102) (98). Confirming an $SDHC$ promoter hypermethylation can help guide long-term management and surveillance protocols and may ultimately enable personalised therapeutic strategies for patients with malignant disease.
**Figure 8.5:** Figure A is an axial CT image showing metastatic wt GIST liver lesions from case #001 and figure B and D shows loss of SDHB protein expression on immunohistochemical analysis of the primary wt GIST tumour in case #001 and #003 respectively. Figure C shows a thoracic PGL in case #021 as demonstrated by the white arrow. Figure E shows a sagittal AP view of the left lung from case #004 with evidence of a 9mm pulmonary chondroma and figure F shows the *ex vivo* metabolomic analysis of the primary wt GIST tumour from case #004 illustrating succinate accumulation at 2.4ppm on the spectra. Figure G demonstrates the histology of a pulmonary chondroma, with evidence of normal collapsed lung tissue illustrated by the white arrow and chondrocytes in the tumor marked by the red arrow. Figure H shows the mixed epithelioid histology of a wtGIST from case #003.
**Figure 8.6:** Illustrates a proposed work flow for the investigation of *SDHC* promoter methylation in a clinical setting for A) PPGL and B) wt GIST.

* = next generation sequencing panel for patients with PPGL including the genes; *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *FH*, *TMEM127*, *RET*, *VHL*, *MAX* and including multiplex ligation dependent probe amplification for deletions and duplication for *VHL*, *SDHB*, *SDHC*, *SDHD*.

** = next generation sequencing panel for patients with wtGIST including the genes; *SDHA,SDHB,SDHC,SDHD,KIT,PDGFRA,NF1* and including multiplex ligation dependent probe amplification for deletions and duplication for *SDHB, SDHC, SDHD*.
8.6: Conclusions

This study has demonstrated that SDHC epimutations can be diagnosed using a pyrosequencing method to assess SDHC promoter methylation from FFPE tumour samples. Improving the accessibility of clinical testing for establishing tumour and constitutional SDHC promoter methylation status is crucial as it makes an important contribution to SDH deficient wtGIST (102) (98). Confirming an SDHC promoter hypermethylation will help guide long-term management and surveillance protocols and may ultimately enable personalised therapeutic strategies for patients with malignant disease.
Chapter 9

Discussion
Chapter 9: Discussion

9.1: The identification of new genotype phenotype correlations

9.2: The implementation of new functional tools to aid the clinical interpretation of $SDHx$ variants of uncertain significance

9.3: The translation of new biomarkers into clinical utility

9.4: The evaluation of potential therapeutic targets in SDH-deficient disease and PPGL

9.5 Conclusions

9.6 Future studies
The investigations reported here represent a ‘multi-omics’ based approach to the study of SDH-deficient disease. The main barriers to optimal clinical practice in the diagnosis of SDH-related neoplasia have previously been discussed in chapter 1 and include; i) frequent detection of novel variants of uncertain significance in \(SDHx\) and the associated challenge of variant interpretation, ii) a lack of robust biomarkers to predict malignant disease and iii) the limited therapeutic options for patients with malignant SDH deficient disease. The aims of this PhD were developed with these important clinical issues in mind and I will now discuss the major findings of my PhD and how these findings could be translated into improved patient care.

9.1: The identification of new genotype phenotype correlations

The recommendations for asymptomatic carriers of a germline pathogenic \(SDHx\) variant include; regular surveillance using cross sectional imaging every two to three years and annual biochemical screening for PPGL (90). However, there is no consensus on the frequency of surveillance or on the optimal radiological modality. Therefore, improving our knowledge of genotype-phenotype correlations can better inform our surveillance practices and enable clinicians to better counsel their patients on future risk.

As part of this PhD, I have investigated the genetic architecture of a renal tumour and PPGL without evidence of VHL disease (non-VHL RAPTAS), occurring in a single individual or a family. This study demonstrated that non-VHL RAPTAS is genetically heterogeneous, with six genes (\(SDHA, SDHB, SDHC, SDHD, TMEM127, MAX\)) now implicated. A pathogenic germline variant was identified in 8/22 (36%) kindred’s with non-VHL RAPTAS included in the study. Importantly, pathogenic germline \(SDHB\) variants were the most common cause of
non-VHL RAPTAS identified in both our study (6/22 kindred’s, 27.3%) and cases reported in the literature (16/21 kindred’s, 76%). Notable genotype-phenotype correlations were identified including; i) a higher than expected proportion of copy number variants (CNV) in \textit{SDHB}-associated RAPTAS, ii) a mean interval of four years between tumour development (range 1-16 years) and iii) the finding that PPGL was the more common presenting phenotype in patients with \textit{SDHB} associated non-VHL RAPTAS in our study and the literature. This study also implicated a germline pathogenic \textit{MAX} variant in the development of RCC and non-VHL RAPTAS for the first time and further consolidated the role of \textit{TMEM127} as an RCC and RAPTAS predisposition gene.

Therefore data from this study would suggest that patients with \textit{SDHB} exonic deletions may have a higher risk of developing RAPTAS (though further studies are required to confirm this finding) and that PPGL is the most common presenting phenotype with a mean interval of 4 years between tumour development in our cohort. This study also supports the value of lifelong surveillance in patients with germline \textit{SDHB} variants, as the longest interval between tumour development was 16 years and two patients with \textit{SDHB} associated non-VHL RAPTAS, developed RCC over the age of 60 years. Finally, this study demonstrated that non-VHL RAPTAS can rarely be caused by pathogenic germline variants in other \textit{SDHx} subunit genes as well as \textit{MAX} and \textit{TMEM127}. 

267
9.2: The implementation of new functional tools to aid the clinical interpretation of SDHx variants of uncertain significance

Missense variants are the most common type of disease causing variant affecting the SDHx subunit genes but assigning pathogenicity to novel SDHx missense variants poses a significant clinical challenge. A primary objective of my PhD studies was to evaluate new functional tools for the assessment of variants of uncertain significance in SDHx genes and three tools have been investigated including i) an in silico prediction model for variants of uncertain significance in the SDHA gene, ii) immunohistochemistry for the detection of the SDHB protein and iii) targeted ex vivo metabolomic analysis using a NMR spectroscopy technique for the detection of succinate accumulation.

The computational prediction of 18 novel missense SDHA variants using DUET, predicted that the majority of variants (13/18, 72.2%) would have a destabilising effect on the protein and facilitated further classification of the variants according to ACMG criteria. However, 75% (18/24) of identified rare (frequency <0.01%) germline SDHA missense variants present in the ESP6500 cohort from Exome Variant Server, were also predicted to be destabilizing by DUET and pathogenic by PolyPhen/SIFT. This analysis highlighted the need to combine protein structure and bioinformatic predictions of pathogenicity with additional functional studies in order to ensure accurate classification of novel SDHx gene variants. Furthermore, the ACMG criteria now also mandate that well established functional analysis is combined with evidence from computational tools in order to assign pathogenicity to novel missense variants(154).
I first investigated SDHB immunohistochemistry (IH) as an adjunctive functional tool for the early detection of SDH deficient disease and for assessing \(SDHx\) variants of uncertain significance. An initial validation study confirmed that SDHB IH was a sensitive test for the detection of SDH deficiency and correlated with the germline genetic status in 19/20 cases included in this validation study. Importantly, no inter-observer variation was noted and this validation study facilitated the introduction of SDHB IH as a clinically available diagnostic test in Cambridge University Hospital in January 2016. SDHB IH was performed on a further 90 tumour samples including 42 non-PPGL tumour samples and when correlated with germline genetic testing demonstrated a sensitivity of 96%. An equivocal SDHB IH result occurred in three cases including two patients with two different known pathogenic variants in \(SDHA\) (c.91C>T p.Arg31Ter and c.1765C>T p.Arg589Trp) and a patient with a pathogenic truncating \(SDHD\) variant (c.296delT, p.Leu99fs). This study demonstrated that SDHB IH is a valuable tool for the detection of SDH deficiency and can be employed to assess variants of uncertain significance in \(SDHx\). Although the sensitivity of this functional tool was estimated at 95-96%, important limitations were identified, including the lower sensitivity of SDHB immunohistochemistry for \(SDHA\) and \(SDHD\) variant detection, which has previously been demonstrated in other studies (7). This study has also demonstrated that the specificity of SDHB IH can be affected by pathogenic somatic or germline \(VHL\) variants, as SDHB IH demonstrated loss of SDHB expression in a RAPTAS case with a phaeochromocytoma harboring a somatic \(VHL\) variant (c.245G>T p.Arg82Leu).

In addition to computational and proteomics based approaches, functional tests exploring SDH enzyme function were explored using a nuclear magnetic resonance (NMR) spectroscopy technique; high resolution magic angle spinning (HRMAS). The mean succinate concentration was 15-fold higher in the SDH deficient tumours (4.7mM SD\(+\)3.13) compared to the \(SDHx\) wild-type tumours (mean succinate 0.31mM, SD\(+\)0.12, \(P=0.0066\)). No
detectable succinate peak was identified in any of the sporadic tumours or tumours caused by a variant in a non-SDHx predisposition gene (e.g. VHL or RET). A succinate concentration of greater than 0.78 mM was identified as being diagnostic of SDH deficiency using receiver operator curve (ROC) analysis. This study demonstrated that targeted metabolomics analysis for succinate accumulation as a marker of SDH enzyme dysfunction is a sensitive tool for the detection of SDH deficiency. A limitation of this approach includes the requirement of fresh frozen tumour samples and the potential for metabolite concentrations to be affected by surgical procedures and storage conditions.

The evaluation of three functional tools for the assessment of SDHx variant pathogenicity has confirmed that a combination of functional and computational analysis is preferable in order to accurately assess putative SDHx missense variants. SDHB immunohistochemistry is cost effective, can be performed on paraffin embedded blocks and this study and others has demonstrated good sensitivity (>95%). Therefore, SDHB IH is a good first line functional tool for the clinical assessment of SDHx variants with the caveats that test sensitivity is reduced for SDHA and SDHD variants and specificity can be affected by germline and somatic VHL variants.

Metabolomic analysis is a very sensitive functional test for the verification of SDHx variant pathogenicity as it provides valuable information on the SDH enzyme function. However, due to the requirements for fresh frozen tissue, metabolomic analysis is likely to have particular application as an adjunct to SDHB IH for confirming novel SDHA and SDHD variant pathogenicity, or in the setting of equivocal results using SDHB IH (Figure 9.1).
**Figure 9.1:** A proposed algorithm for the assessment of a variant of uncertain significance in *SDHx.*

*= Loss of heterozygosity of novel *SDHx* variant

9.3: The translation of new biomarkers for phaeochromocytoma/paraganglioma and SDH deficient disease into clinical utility

In 2017, the WHO classification of adrenal tumours abolished the term ‘benign’ for PPGL, stating that all PPGLs should be considered to have metastatic potential (328). Therefore the evaluation of new and robust biomarkers to predict malignant potential and tumour recurrence is an important clinical priority for patients with PPGL and SDH deficient disease.

At present for PPGL, tumour size (>5cm), local invasion and the detection of a germline SDHB variant are the best predictors of malignant or recurrent disease risk (329). As tumour evolution will differ from one individual to the next, it is important to remember that an individual biomarker may not be ubiquitous and that a combination of predictive biomarkers or an algorithm based on clinical phenotype, pathology, secretory phenotype as well as germline and somatic molecular profiling, may enable a more reliable prediction of malignant disease and therapeutic response.

In this study, I investigated the somatic molecular profiles of 70 PPGL, with the aim of determining whether somatic profiling could be utilised for additional prognostic and therapeutic utility in clinical practice. A potential somatic driver variant was identified and validated in 19% (13/70). Aside from a single case of hereditary metastatic PGL (case #18), somatic driver variants were identified exclusively in sporadic PPGL. The observation that somatic driver variants are more frequent in sporadic versus hereditary PPGL in this study and others (210) would suggest that somatic molecular profiling may be best utilised as a potential biomarker in sporadic PPGL.

Similar to the COSMIC (https://cancer.sanger.ac.uk/cosmic) somatic variant frequency data for PPGL, variants in NF1 (3/70, 4.2%), VHL (3/70, 4.2%) and HRAS (2/70, 2.8%) were among the most common identified in this study. No case of metastatic PPGL with a somatic
driver variant in HRAS, VHL or NF1 was identified and only rare reports exist in COSMIC (https://cancer.sanger.ac.uk/cosmic) and the literature (330) (210). This finding might suggest that identifying a somatic driver variant in NF1, VHL or HRAS in combination with other clinical parameters including tumour size, histology and tumour location (eg. adrenal versus extra-adrenal), could be used to predict a lower risk of malignant potential and inform a less comprehensive and frequent surveillance strategy. Similarly, somatic variants in the ATRX gene (222) and more recently structural rearrangements in the TERT gene (330) identified by whole genome sequencing, have been implicated as molecular drivers in malignant PPGL. However larger prospective studies are needed to validate the application of somatic molecular profiling as a prognostic test in PPGL.

In addition to predicting the malignant potential of PPGL, somatic molecular profiles may also predict the most suitable surveillance strategies for PPGL recurrence for example; nuclear imaging modalities such as^{18}F-FDG (208) and ^{68}\text{Gallium-DOTATATE PET CT} (288) have superior diagnostic sensitivity in patients with SDHx and VHL germline variants and ^{18}F-DOPA PET CT is superior in patients with gene variants affecting kinase signalling pathways e.g. NF1 and HRAS (329).

Predictive biomarkers of therapeutic response are valuable in clinical practice and in clinical trial settings. Historically, cross sectional imaging using CT or MRI were most commonly employed to determine therapeutic efficacy. However, it is now increasingly evident that interval cross sectional imaging studies have significant limitations, particularly in monitoring response to targeted therapies in rare and heterogeneous tumours. In this study, targeted in vivo metabolomic profiling using MRI spectroscopy (^{1}\text{H-MRS}) demonstrated potential translational utility as a non-invasive biomarker in the precision management of SDH-deficient disease. Serial ^{1}\text{H-MRS} in a patient with metastatic SDH deficient PGL, showed that in vivo measurement of succinate using ^{1}\text{H-MRS} could be used as a biomarker of
successful treatment response. The benefits of $^1$H-MRS include high test specificity and avoidance of exposure to ionizing radiation. This study has demonstrated that $^1$H-MRS could be used alone or in combination with existing functional imaging tests such as $^{18}$F-FDG PET-CT to monitor therapeutic response. The future application of $^1$H-MRS may be particularly relevant in a clinical trial setting to monitor biological response to experimental treatments in SDH deficient disease (e.g. demethylating agents in patients with an SDHC epi-mutation).

**9.4: The evaluation of potential therapeutic targets in SDH-deficient disease and PPGL**

In this study, somatic molecular profiling of PPGL demonstrated that somatic mutations in genes involved in cell signaling are commonly implicated in sporadic PPGL. Although malignant disease is rarely associated with somatic variants in genes ($NF1$, $HRAS$, $VHL$) involved in this pathway, altered cell signaling may render these tumours more sensitive to targeted treatment with tyrosine kinase inhibitors ($VHL$), ERK1/2 inhibitors ($RAS$) (242) or MEK inhibitors ($NF1$) (331). The novel discovery of a truncating somatic $FBXW7$ variant in a single case of a metastatic phaeochromocytoma in this study requires further investigation to determine if loss of function of $FBXW7$ in this tumour may predict response to treatment with mTOR inhibitors.

The metabolic vulnerability of SDH deficient tumours provides an opportunity for therapeutic targeting. In this study untargeted *ex-vivo* metabolomic profiling identified that SDH deficient tumours have a specific metabolomic fingerprint compared to non-SDHx mutated tumours, with lower concentrations of metabolites involved in amino acid and membrane phospholipid metabolism. Metabolic reprogramming in SDH deficient tumours extended to involve amino acid intermediates such as aspartate, which was identified as the next most sensitive (100%) and specific (81%) metabolite after succinate, at a concentration
of < 2.41 mM on ROC curve analysis, for differentiating SDHx from non SDHx mutated tumours. Aspartate is essential for DNA synthesis and cellular proliferation and recent studies have suggested that SDH deficient tumours overcome the deficiency in aspartate by upregulating the enzyme pyruvate carboxylase (271). Indeed, in this study, a lower concentration of branch chain amino acids (alanine, valine and leucine) in SDH deficient tumours would support the hypothesis that pyruvate metabolism is being preferentially diverted to carboxylation reactions via pyruvate carboxylase in SDH deficient tumours. The hypothesis that pyruvate carboxylase upregulation in SDH deficient tumours is a potential therapeutic target has been further strengthened by a recent study demonstrating that knock down of pyruvate carboxylase in a SDH deficient kidney cell model was associated with significantly reduced cellular proliferation compared to the pyruvate carboxylase intact SDH deficient cells (332). This finding correlated with the histopathological finding that pyruvate carboxylase expression on immunohistochemistry was significantly greater in areas of higher proliferation in SDHB-mutated RCC compared to areas of low-proliferation in the same tumour (332). Intriguingly, pyruvate carboxylation was also recently identified as the main anapleortic reaction in non-small cell lung cancer (NSCL) and silencing of pyruvate carboxylase expression also affected NSCL proliferation in an ex-vivo and mouse xenograft models in one study (333). Identifying this metabolic vulnerability in SDH deficient tumours and other malignancies, has encouraged the development of new therapies targeting metabolic adaptations in cancer and the recent description of the pyruvate carboxylase allosteric binding site may facilitate the future development of a therapy directed at modulating pyruvate carboxylase activity (334).

Targeted ex-vivo and in vivo metabolomic profiling of PPGL and GIST tumours in this study has identified tumours with elevated succinate levels and untargeted ex-vivo analysis has also identified a PGL with 2-hydroxyglutarate accumulation and an occult somatic IDH1
pathogenic variant. Detection of oncometabolite accumulation is relevant as recent studies would suggest that accumulation of either succinate, fumarate or 2-hydroxyglutarate (2-HG) may render tumours susceptible to synthetic-lethal targeting with poly(ADP)-ribose polymerase (PARP) inhibitors (303)(335). Accumulation of these oncometabolites cause inhibition of α-ketoglutarate-dependent dioxygenases and the subsequent inhibition of two key lysine demethylases; KDM4A and KDM4B, which ultimately dysregulates DNA homologous repair (303). Therefore, in the future ex vivo and in vivo metabolomic analysis for oncometabolite accumulation as demonstrated in this study, may provide important prognostic information regarding the potential efficacy of PARP inhibitors in patients with metabolically driven tumours.

In addition to a unique metabolome, SDH deficient tumours can also be characterized by their unique methylome, namely a global hypermethylation phenotype (102)(83). Therefore de-methylating agents such as azacytidine and decitabine may be an effective therapeutic option and clinical trials are currently underway to further evaluate this (ClinicalTrials.gov Identifier: NCT03165721). De-methylating agents may be particularly effective for tumours harboring an SDHC epimutation. Although the biological consequences of SDHC hypermethylation and germline SDHx variants appear to be similar, SDHC epimutations are potentially reversible. Although the diagnosis of an SDHC epimutation may influence therapeutic strategies as well as informing appropriate surveillance strategies, testing for an SDHC epimutation is not routinely available in clinical practice. In this study, a pyrosequencing based assay was optimized for detection of SDHC promoter hypermethylation. This study demonstrated that the methylation status of 12 CpG’s in CpG27 in the promoter region of the SDHC gene could be accurately assessed and that detection of hypermethylation of the SDHC promoter correlated with reduced SDHC mRNA expression. SDHC promoter region methylation was identified in 6/15 patients with SDH deficient
wtGIST but no case of PPGL (0/17). This study has; i) contributed to a better understanding of the phenotype associated with an SDHC epi-mutation, ii) validated a diagnostic assay, which can be translated into clinical utility for the detection of an SDHC epi-mutation and iii) facilitated the provision of recommendations for testing, with the objective of identifying those patients with SDH deficient disease, who may benefit most from de-methylating therapies.

**9.5 Conclusions**

This PhD study has contributed to a better understanding of genotype-phenotype correlations in SDH deficient disease and has facilitated the translation of new techniques into clinical utility which will enable variants of uncertain significance in SDHx to be more accurately assessed for pathogenicity. The combined investigation of the metabolome, epigenome and tumour genome of SDH deficient tumours and PPGL has facilitated a better understanding of tumour biology and may contribute to a precision medicine approach to diagnosis, disease surveillance, and therapeutic selection in the future for patients with PPGL and SDH deficient disease.
9.6 Future studies

I plan to apply the information gained from early preliminary metabolomic analysis in this study to focus a more detailed investigation of enzyme flux in SDH deficient tumours.

Hyperpolarised MRI enables stable isotopes such as $^{13}$C-pyruvate to be detected by magnetic resonance spectroscopic imaging by temporarily redistributing the populations of nuclear spins in a magnetic field through a process termed ‘hyperpolarisation’. This technique can then provide the necessary information about tissue biochemistry *in vivo* providing a dynamic insight into the tumour biochemistry. The focus on pyruvate carboxylase (PC) is based on the hypothesis that PC is essential in SDH deficient cells to enable production of oxaloacetate, aspartate and create biomass for cellular proliferation. Thus *in vivo* detection of pyruvate conversion to oxaloacetate by irreversible carboxylation may help stratify those SDH deficient tumours with highest malignant potential and confirm whether SDH deficient cells are dependent on PC to proliferate, facilitating future studies investigating therapeutic targeting of PC.

Expertise with hyperpolarised MRI exists in this institution and within this collaborative research group. Cambridge is one of only five facilities in the world capable of undertaking clinical hyperpolarised MRI imaging, strengthening the value of this project as a unique research opportunity.

Future studies such as this, could provide essential data necessary to initiate collaboration with local industry to identify potential therapies directed against identified vulnerable metabolic targets in SDH deficient tumours
Chapter 10

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Chapter 11
Appendix
Is there an optimal preoperative management strategy for phaeochromocytoma/paraganglioma?

B.G. Challis*†, R.T. Casey†, H.L. Simpson† and M. Gurnell*†

*Metabolic Research Laboratories, Wellcome Trust–MRC Institute of Metabolic Science, ‡Department of Medical Genetics, National Institute for Health Research Biomedical Research Centre, and †Wolfson Diabetes and Endocrine Clinic, Institute of Metabolic Science, Addenbrooke’s Hospital, Cambridge, UK.

Summary

Phaeochromocytomas and paragangliomas (PPGLs) are catecholamine secreting neuroendocrine tumours that predispose to haemodynamic instability. Currently, surgery is the only available curative treatment, but carries potential risks including hypertensive and hypotensive crises, cardiac arrhythmias, myocardial infarction and stroke, due to tumoral release of catecholamines during anaesthetic induction and tumour manipulation. The mortality associated with surgical resection of PPGL has significantly improved from 20–45% in the early 20th century (Appgar & Papper, AMA Archives of Surgery, 1951, 62, 634) to 0–2.9% in the early 21st century (Kinney et al. Journal of Cardiothoracic and Vascular Anesthesia, 2002, 16, 359), largely due to availability of effective pharmacological agents and advances in surgical and anaesthetic practice. However, surgical resection of PPGL still poses significant clinical management challenges. Preoperatively, alpha-adrenoceptor blockade is the mainstay of management, although various pharmacological strategies have been proposed, based largely on reports derived from retrospective data sets. To date, no consensus has been reached regarding the ‘ideal’ preoperative strategy due, in part, to a paucity of data from high-quality evidence-based studies comparing different treatment regimens. Here, based on the available literature, we address the Clinical Question: Is there an optimal preoperative management strategy for PPGL?

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Correspondence: Dr Mark Gurnell, Metabolic Research Laboratories, Wellcome Trust–MRC Institute of Metabolic Science, Box 289, Level 4, Addenbrooke’s Hospital, Cambridge CB2 0QQ, UK. Tel.: +44 (0) 1223 245151; E-mail: mg299@medschl.cam.ac.uk

†Dr Helen Simpson, Wolfson Diabetes and Endocrine Clinic, Institute of Metabolic Science, Box 281, Addenbrooke’s Hospital, Cambridge CB2 0QQ, UK. Tel.: +44 (0) 1223 348799; E-mail: hls41@medschl.cam.ac.uk

*These authors contributed equally to this work.

What are the goals of preoperative pharmacological therapy?

At a headline level, normalization of blood pressure and heart rate, and restoration of intravascular fluid status are the main objectives of preoperative pharmacological management. Current guidelines suggest adrenergic blockade should be initiated 7–14 days prior to surgery. However, the average duration of treatment varies depending on the regimen adopted, and whether inpatient or outpatient therapy is initiated. Most centres report an average preoperative treatment duration of 2–6 weeks. Although in some institutions treatment may be started even earlier, there is no evidence to suggest that additional benefit is derived from longer preoperative blockade. Similarly, there is no consensus regarding haemodynamic thresholds that signal adequate blockade, with current published recommendations based largely on noncontrolled studies and institutional experience. Roizen et al. proposed several indicators of adequate preoperative alpha-blockade which included: (i) No in-hospital blood pressure >160/90 mmHg for 24 h prior to surgery; (ii) No orthostatic hypotension with blood pressure <80/45 mmHg; (iii) No ST segment or T wave ECG changes for 1 week prior to surgery; (iv) No more than five premature ventricular contractions per minute. Subsequently, others have suggested a lower preoperative blood pressure (<130/80 mmHg while seated; systolic BP >90 mmHg on standing) and controlled heart rate (60–70 beats per minute while sitting), which align with current Endocrine Society recommendations. However, whether preoperative normalization of systolic blood pressure is mandatory in all patients has been questioned. Lentschener et al. have proposed that only patients with hypertension-induced organ dysfunction require systolic blood pressure normalization prior to surgery, based on their findings that high preoperative systolic BP per se is not predictive of perioperative haemodynamic instability.

Do all patients with PPGL require preoperative hypotensive drugs?

Current consensus recommends that all hypertensive patients with biochemically confirmed PPGL should receive preoperative pharmacological management. Similarly, in patients with...
functional PPGL who are apparently normotensive and asymptomatic, tumour manipulation may provoke an increase in blood pressure and preoperative medical management is therefore recommended. Preoperative medical treatment may not be required for patients with nonfunctioning (defined by negative metanephrine screening) parasympathetic-derived head and neck parangliomas or those with exclusive dopamine-secreting tumours. However, intraoperative anaesthetic vigilance and expertise are still required.

Preoperative blockade with alpha-adrenoceptor antagonists is also standard care for management of PPGL in pregnancy. Phenoxybenzamine (PBZ) is the preferred agent and is safe for the fetus; however, blood pressure control must be carefully monitored to ensure adequate placental perfusion. The optimal timing for surgical resection of a PPGL during pregnancy is generally considered to be in the second trimester, thereby allowing the pregnancy to progress to normal term/delivery thereafter. However, if this is not possible, then treatment with PBZ should continue until the fetus has reached a satisfactory weight; careful discussion between the patient, endocrinologist, obstetrician and anaesthetist regarding the timing of tumour resection and delivery of the baby by Caesarean section will be required.

### Which preoperative hypotensive drugs have been used?

#### Alpha-adrenoceptor antagonists

Phenoxybenzamine (PBZ), a noncompetitive α1- and α2-adrenoceptor antagonist, is the most widely used agent for preoperative blockade. Owing to formation of a permanent covalent bond with α-adrenoceptors, PBZ has a long duration of action (t½ = 24 h, which tapers following synthesis of new receptors), and may contribute to sustained hypotension following tumour removal. Side-effects include nasal congestion, CNS sedation, orthostatic hypotension, reflex tachycardia and, at higher doses, paradoxical hypertension. The starting dose is 10 mg twice daily with a recommended maximum daily dose of 1 mg/kg, and average dose requirement of 40–60 mg/day. For the majority of patients, pretreatment with PBZ can be undertaken on an outpatient basis. It is important to note, however, that intraoperative hypertensive surges (systolic blood pressure >160 mmHg) may still occur in patients deemed to be adequately pretreated with PBZ (Fig. 1a,b). In addition, high cost and restricted availability preclude routine use of PBZ in some centres and countries.

Compared with PBZ, selective α1-adrenoceptor antagonists such as prazosin, terazosin or urapidil, have short half-lives due to competitive inhibition and displacement by endogenous catecholamines. The shorter half-life of selective α1-adrenoceptor antagonists results in less reflex tachycardia and a shorter duration of postoperative hypotension. In contrast, modified release doxazosin has a longer duration of action (t½ = 16–36 h), allowing once daily dosing as well as dose optimization in the days prior to surgery. In general, doxazosin does not cause reflex tachycardia or significant postoperative hypotension.

Several retrospective studies have reported the benefit of preoperative blockade with PBZ using end-points such as operative mortality, intraoperative blood pressure excursions and postoperative complications. There are no published randomized clinical trial data comparing PBZ with selective alpha-blockade. One retrospective study found no difference in blood pressure or intraoperative/postoperative fluid requirements between patients pretreated with PBZ vs doxazosin or prazosin. Another retrospective multicentre study reported higher postoperative inotropic requirements in patients pretreated with PBZ and higher intraoperative blood pressure readings in those who received doxazosin.

Evidence for efficacy of selective α1-adrenoceptor antagonists in the preoperative management of PPGLs exists mainly for doxazosin (DX). In one study DX performed as well as PBZ with respect to intraoperative haemodynamic stability, with fewer reported side-effects, episodes of intraoperative tachycardia and postoperative fluid requirements, and no difference in mortality. In contrast, other groups observed that pretreatment with DX resulted in higher systolic blood pressures before and after anaesthetic induction compared with PBZ. Van der Zee et al. recently reviewed studies comparing pretreatment with PBZ vs DX, and concluded that there was no evidence to suggest superiority of one agent over the other, and that alpha-adrenoceptor blockade per se was efficacious. In another retrospective series, preoperative treatment with prazosin was associated with no deaths, although significant intraoperative hypertensive surges occurred in 83% of treated patients. Successful surgical outcomes following preoperative urapidil administration have also been reported. However, hypertensive surges occurred at induction and/or tumour manipulation in all patients. Esmolol administration was required to control intraoperative tachycardia in one-third of cases.

One retrospective study reported no benefit of preoperative alpha-blockade in normotensive patients with secretory PPGL. It is important to note that the number of subjects in the treatment group was almost twice that of the control group and that a modest dose of DX (4 mg) was used in the treatment group. No difference was seen in intraoperative blood pressure in patients treated with DX compared with patients who did not receive alpha-adrenoceptor blockade. There was, however, increased administration of intraoperative inotropes and colloid in the DX-treated group.

#### Beta-adrenoceptor antagonists

Beta-adrenoceptor antagonists are contraindicated in the absence of effective α1-receptor blockade due to the risk of a potentially fatal hypertensive crisis secondary to unopposed alpha-adrenoceptor stimulation. Preoperative use of β-blockers is generally reserved for prevention and treatment of cardiac arrhythmias and reflex tachycardia, and no evidence exists to support the routine use of beta-blockade in the management of noradrenaline-secreting tumours in the absence of arrhythmias. However, preoperative use of β-blockers should be considered in the management of tachycardia or tachyarrhythmias induced by adrenaline-secreting PPGL. Cost and dosing schedules may need
to be considered when choosing a beta-adrenoceptor antagonist (the latter to maximize compliance).

**Calcium channel antagonists**

Calcium channel antagonists (CCB) inhibit noradrenaline-mediated calcium influx into vascular smooth muscle thereby inducing coronary and peripheral artery relaxation to control hypertension, tachyarrhythmias and possibly coronary vasospasm.\(^1\) These agents cause minimal hypotension and may be best suited for normotensive patients with paroxysmal hypertension or intolerance to alpha-adrenoceptor antagonists.\(^6\)

Brunaud \textit{et al.} compared patients treated with nifedipine with patients treated with PBZ and beta-blockade and found that intraoperative mean systolic blood pressure and incidence/duration of hypertensive surges was lower in PBZ-treated patients.\(^25\) However, postoperatively, PBZ-treated patients had an increased incidence of hypotension and greater fluid requirements. No difference in overall haemodynamic stability was observed between groups.\(^25\) Similarly, Siddiqi \textit{et al.} reported no difference in

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\(^{1}\) Brunaud \textit{et al.}

\(^{25}\) Siddiqi \textit{et al.}

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Fig. 1 Intraoperative haemodynamic changes in three patients undergoing adrenalectomy for phaeochromocytoma. (a) 68-year-old man (70 kg): plasma metadrenaline 1283 pmol/l (RR: 0–600) and normetadrenaline 1086 pmol/l (RR: 0–1000). Computed tomography (CT) imaging revealed a 9.0 × 4.5 cm phaeochromocytoma. Preoperative blockade was established with phenoxybenzamine (PBZ) over a 5-week period (maximum tolerated dose 20 mg twice daily) and propranolol (10 mg thrice daily) in accordance with published guidelines.\(^1\) A minimum 2.5 l oral fluid intake per day was advised while taking PBZ. He also received 5 l of 0-9% sodium chloride intravenously in the 48-h period prior to surgery. There were no postoperative complications. (b) 72-year-old man (85 kg): plasma metadrenaline 427 pmol/l (RR: 0–600) and plasma normetadrenaline 17 187 pmol/l (RR: 0–1000). CT revealed a 12.5 × 11.5 cm right-sided phaeochromocytoma. Preoperative blockade was established with PBZ over a 7-week period (maximum tolerated dose 20 mg twice daily). As the patient was clinically hypovolemic at initiation of PBZ treatment, 3 l of oral fluid intake per day was supplemented with 2 l of 0-9% sodium chloride intravenously as an outpatient on our endocrine day unit, and a further 6 l of intravenous fluid in the 48 h preoperatively. There were no postoperative complications. (c) 75-year-old man (65-8 kg): plasma metadrenaline >18 000 pmol/l (RR: 0–600) and plasma normetadrenaline 10 120 pmol/l (RR:0–1000). CT revealed a 5 × 7 cm right-sided phaeochromocytoma. The patient declined medical or surgical management. However, shortly afterwards he presented with acute small bowel obstruction necessitating emergency surgery in the absence of preoperative blockade. A right adrenalectomy was performed during the same procedure. There were no postoperative complications. Art line, arterial line; HR, heart rate; IBP, invasive blood pressure; NIBP, noninvasive blood pressure; solid arrow indicates time of anaesthetic induction/intubation; dashed arrow signifies the point at which the PPGL was removed. [Colour figure can be viewed at wileyonlinelibrary.com]
haemodynamic stability between patients treated with either nicardipine or PBZ, although patients pretreated with the former had a smaller mean tumour size and lower metanephrine levels.\(^{26}\) Finally, in another retrospective series, nicardipine monotherapy was associated with low mortality rates but increased incidence of intraoperative hypertensive episodes.\(^{27}\)

**x-methyl-para-tyrosine (Metyrosine)**

Metyrosine competitively inhibits tyrosine hydroxylase, the enzyme that regulates the rate-limiting step of catecholamine biosynthesis,\(^{14}\) to reduce catecholamine levels. Metyrosine is most often used in conjunction with alpha-blockade, and in combination may reduce both intraoperative haemodynamic instability and postoperative cardiovascular morbidity. However, high cost, limited availability and intravenous route of administration restrict routine use. Metyrosine has been reported to provide improved haemodynamic stability and reduced postoperative fluid requirements, although no differences in surgical outcome.\(^{28–30}\) It is important to note, however, that hypertensive crises may still occur with metyrosine monotherapy.\(^{31}\)

**When should add-on therapy be considered?**

Add-on therapy should be considered when blood pressure is not adequately controlled with a single agent or the patient is intolerant of escalating doses of monotherapy. In either setting, metyrosine or CCB can be used effectively as add-on therapies to alpha-adrenoceptor antagonists and, in combination, have been found to provide superior haemodynamic stability in some studies.\(^{26,28}\) Add-on therapy should also be used to treat tachycardia or cardiac arrhythmias, with beta-adrenoceptor antagonists the preferred agents.

**When should preoperative alpha-blockade be discontinued?**

Limited data exist to inform this decision and discontinuation of treatment the night prior vs the morning of surgery is guided by the choice of alpha-blockade and half-life of the agent. For example, PBZ has a longer half-life and in patients scheduled for an early morning theatre slot treatment is generally continued until the evening prior to surgery; however, this approach is not universal with some clinicians advising a final dose on the morning of surgery. Where a selective alpha-blocker with a shorter duration of action is used, the last dose is usually administered on the day of surgery.

**Do all patients require preoperative fluid replacement?**

There is no randomized controlled evidence to support a role for routine preoperative fluid replacement. However, retrospective data suggest that fluid and salt replacement may limit postural hypotension and postoperative hypotension\(^3\) by optimizing intravascular status. If patients are unable to tolerate a high fluid intake orally, administration of intravenous fluids for 24–48 h before surgery is often advised. However, the requirement for preoperative intravenous fluid has been queried, as Lentschener et al.\(^7\) observed no difference in mortality when intravenous fluids were given on an ‘as needed’ basis only, as guided by arterial blood pressure, indicating that ‘prophylactic’ administration of intravenous fluids may not improve outcomes in PPGL surgery when appropriate anaesthetic expertise is readily available.

**What is the value of perioperative management?**

Even when preoperative blockade is carefully managed, and optimal alpha-adrenoceptor blockade and fluid replacement is deemed to have been achieved, intraoperative haemodynamic instability can still occur as illustrated in Fig. 1a,b. Moreover, in some instances, such as emergency surgery in patients with a known phaeochromocytoma, it may not be possible to establish adequate preoperative blockade prior to surgery; however, safe clinical outcomes can still be achieved. Fig. 1c illustrates such a case, suggesting that perioperative management may actually be more critical for achieving good clinical outcomes than administration of perioperative hypotensive drugs. This thesis is supported by recent reports which reason that adequate control of intraoperative hypertension can be achieved through meticulous blood pressure monitoring, careful surgical practice and administration of fast-acting hypotensive agents when necessary.\(^32,33\) Consistent with this, several studies have shown that in the perioperative period, continuous blood pressure monitoring, administration of vasoactive and anti-arrhythmic drugs, and careful fluid management all contribute to improved patient outcomes.\(^7,10,11\)

**Conclusions**

There is a lack of available randomized clinical trial data to support decision-making on preoperative management of PPGL. Currently, however, ‘PRESCRIPT’ (see clinicaltrials.gov), a randomized, multi-centre open label clinical trial is recruiting subjects to determine whether preoperative treatment with PBZ or DX is superior with regards to minimizing intraoperative haemodynamic instability. Until these data are reported, current recommendations and available evidence support PBZ (or, where not available, DX) as first line preoperative pharmacological therapy in patients with PPGLs. In the majority of cases, a short period of preoperative blockade with PBZ, combined with active fluid management, allows surgery to proceed uneventfully. However, even when the patient’s clinical status is deemed to have been ‘optimized’ prior to surgery, significant intraoperative blood pressure excursions may still occur. There is growing evidence that perioperative anaesthetic expertise is critical for successful management of patients with PPGL undergoing surgery, and we believe that this may in fact be the single most important factor governing outcome.

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Disclosures

No conflicts of interest to disclose for any of the authors.

References

SDHA related tumorigenesis: a new case series and literature review for variant interpretation and pathogenicity

Ruth T. Casey¹,², David B. Ascher³,⁴, Eleanor Rattenberry⁵, Louise Izatt⁶, Katrina A. Andrews¹, Helen L. Simpson², Benjamem Challis², Soo-Mi Park¹, Venkata R. Bulusu⁷, Fiona Laloo⁸, Douglas E. V. Pires⁹, Hannah West¹, Graeme R. Clark¹, Philip S. Smith¹, James Whitworth¹, Thomas G. Papathomas¹⁰, Phillipe Taniere¹¹, Rosina Savisaar¹², Laurence D. Hurst¹², Emma R. Woodward⁵,⁸ & Eamonn R. Maher¹

¹Department of Medical Genetics, University of Cambridge and NIHR Cambridge Biomedical Research Centre, Cambridge CB2 2QQ, UK
²Department of Endocrinology, University of Cambridge and NIHR Cambridge Biomedical Research Centre, Addenbrooke’s Hospital, Cambridge CB2 2QQ, UK
³Department of Biochemistry, University of Cambridge, Sanger Building, 80 Tennis Court Road, Cambridge CB2 1GA, UK
⁴Department of Biochemistry, Bio21 Institute, University of Melbourne, Melbourne, Victoria 3010, Australia
⁵West Midlands Region Genetics Service, Birmingham Women’s Hospital, Birmingham, UK
⁶Department of Medical Genetics, Guy’s Hospital, London, UK
⁷Oncology Centre, Cambridge University Hospitals, Cambridge CB2 2QQ, UK
⁸Manchester Centre for Genomic Medicine, St Mary’s Hospital, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, UK
⁹Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte 30190-002, Brazil
¹⁰Department of Histopathology, King’s College Hospital, London, UK
¹¹Histopathology and Cellular Pathology, University Hospitals Birmingham NHS Foundation Trust, Queen Elizabeth Hospital, Birmingham, UK
¹²The Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

Keywords
Pathogenesis, SDHA, variant

Abstract

Purpose
To evaluate the role of germline SDHA mutation analysis by (1) comprehensive literature review, (2) description of novel germline SDHA mutations and (3) in silico structural prediction analysis of missense substitutions in SDHA.

Patients and methods
A systematic literature review and a retrospective review of the molecular and clinical features of patients identified with putative germline variants in UK molecular genetic laboratories was performed. To evaluate the molecular consequences of SDHA missense variants, a novel model of the SDHA/B/C/D complex was generated and the structural effects of missense substitutions identified in the literature, our UK novel cohort and a further 32 “control missense variants” were predicted by the mCSM computational platform. These structural predictions were correlated with the results of tumor studies and other bioinformatic predictions.

Results
Literature review revealed reports of 17 different germline SDHA variants in 47 affected individuals from 45 kindreds. A further 10 different variants in 15 previously unreported cases (seven novel variants in eight patients) were added from our UK series. In silico structural prediction studies of 11 candidate missense germline mutations suggested that most (63.7%) would destabilize the SDHA protomer, and that most (78.1%) rare SDHA missense variants present...
inherited PCC/PGL/HNPGL and mutations in SDHA
encephalopathy syndrome (Leigh syndrome) about 15 years were associated with autosomal recessive metabolic
Phaeochromocytoma (PCC) is a catecholamine secreting tumor arising from chromaffin tissue in the adrenal medulla. Similar tumors arising from sympathtic ganglion cells outside the adrenal are termed a paraganglioma (PGL) and are to be distinguished from head and neck paragangliomas (HNPGL), which are, in general, nonfunctional tumors arising from parasympathetic ganglia (Boulpaep et al. 2003). PCC/PGL are the most often inherited neuroendocrine tumors with approximately 40% of all cases (NF1, RET, VHL, SDHB, SDHC, SDHD, SDHA, SDHAF2, MAX, TMEM127, HIF2A, MDH2) (Yang et al. 2015; Lorenzo et al. 2013; Burnichon et al. 2010; Qin et al. 2010; Hao et al. 2009; Astuti et al. 2001a, 2001b; Baysal et al. 2000; Niemann and Müller 2000). In nonsyndromic cases of PCC/PGL, germline mutations are most frequently identified in SDHB and SDHD (Jafri et al. 2013; Neumann et al. 2004; Gimenez-Roqueplo et al. 2003; Astuti et al. 2001a,b; Baysal et al. 2000), which encode the B and D subunits of the succinate dehydrogenase (SDH) complex (type II mitochondrial complex) which is an integral component of the citric acid cycle (Oyedotun and Lemire 2004). SDH facilitates the conversion of succinate to fumarate ensuring cellular metabolism of lipids, glucose and amino acids, and feeds into the mitochondrial respiratory chain to generate cellular energy (Oyedotun and Lemire 2004). Mutations in SDHB and SDHD have, in addition to PCC/PGL, also been associated with predisposition to HNPGL, renal cell carcinoma (RCC), gastrointestinal stromal tumors (GIST), and pituitary adenomas (PA) (Pantaleo et al. 2013; Xekouki and Stratakis 2012; Rickets et al. 2010; Astuti et al. 2001a,b; Baysal et al. 2000).

The SDHD protein, together with SDHC, anchors the SDH complex to the inner mitochondrial wall and binds to SDHB, which in turn binds to SDHA (the catalytic subunit of the complex) (Oyedotun and Lemire 2004). Although mutations in SDHB, SDHC, and SDHD were associated with inherited PCC/PGL/HNPGL and mutations in SDHA were associated with autosomal recessive metabolic encephalopathy syndrome (Leigh syndrome) about 15 years ago (Horvath et al. 2006), the association of pathogenic germline SDHA mutations with inherited PCC was described only 5 years ago (Burnichon et al. 2012). In order to better characterize the genetic and clinical features of germline SDHA mutations, we undertook a literature review, and analyzed the clinical and molecular features of 15 new cases that found to have a germline SDHA variant via diagnostic testing and studied, computationally, the effect of novel and previously reported SDHA missense variants on SDHA structure. In addition, we assessed whether mutations might be predicted to disrupt splicing (Di Gaicomo et al. 2013; Woolfe et al. 2010; Wu and Hurst 2016; Pagani et al. 2005; Soukarieh et al. 2016), either by disrupting splice sites or by affecting exonic splice enhancers (Ke et al. 2011; Caceres and Hurst 2013) or silencers (Ke et al. 2011).

Material and Methods

Case series

Details of rare potentially pathogenic germline SDHA (OMIM: 600857, reference sequence: NG_012339.1) variants detected at UK NHS molecular diagnostic laboratories were obtained from those undertaking genetic testing. Referral data were collated on a standardized proforma and included: gender, age at presentation, method of presentation (sporadic vs. familial), location of tumor, presence of bilateral/multifocal disease, and evidence of malignancy. Malignancy was defined as the presence of distant or local regional metastasis. Patients gave written informed consent to a research ethics committee approved research study and/or data was collected as part of a molecular genetics service evaluation study.

Literature review

A SDHA mutation search in association with PCC/PGL, GIST, RCC, PA, Leigh syndrome, and optic atrophy was performed. This search was performed using the Human Gene Mutation Database (www.hgmd.cf.ac.uk), the Leiden Open Variation Database (http://www.lovd.nl/3.0/home),
Assessment of variant pathogenicity

In cases where the detected SDHA variant identified was novel and suspected to be causative in the disease phenotype, classification of variant pathogenicity was performed based on the recently published classification system by the American College of Genomic Medicine (ACMG) (Richards et al. 2015). This system categorizes variants as pathogenic or benign. If a variant does not meet the criteria for either a pathogenic or a benign variant, the recommendation is that this variant be defaulted to a variant of uncertain significance. Within the pathogenic category, variants can be further subclassified as either; pathogenic or likely pathogenic. Similarly within the benign category, sub classification includes benign or likely benign (Richards et al. 2015).

The criteria used to classify a variant included: review of the disease phenotype, the use of the predictive tools SIFT and Poly-Phen2 and when available, review of functional tumor studies (including immunohistochemical staining (IHC) of the SDHB/SDHA protein and loss of heterozygosity studies (LOH)). The presence of the disease allele in a healthy control population was also confirmed by searching the EXAC database (http://exac.broadinstitute.org/). Variants identified in the literature which, were not considered to be disease causing by the authors were excluded.

Information from computational predictive tools above and functional studies when available was combined with in silico predicted changes in protein stability and protein-protein affinity upon mutation for 18 missense variants identified. This information was compiled and variants were classified as per the ACMG recommendations. An online genetic variation tool predictor (http://medschool.umd.edu/Genetic_Variant_Interpretation_Tool1.html) based on ACMG guidelines was used to tabulate the evidence for the 18 missense variants (see Table 2).

Modeling of the SDHA/B/C/D complex and prediction of the effects of missense substitutions

A molecular model of SDHA was generated using Modeller and Macro Model (Schrodinger, New York, NY) using the X-ray crystal structures of Succinate dehydrogenase flavoprotein subunit from the Avian respiratory complex ii (92% sequence identity; PDB ID: 1YQ4) (Huang et al. 2006) and the Flavoprotein subunit of Complex ii from Ascaris suum (72% sequence identity; PDB ID: 3VR8) (Shimizu et al. 2012). The models were then minimized using the MMF94s forcefield in Sybyl-X 2.1.1 (Certa L.P, St Louis, MO, USA), with the final structure having more than 95% of residues in the allowed region of a Ramachandran plot. The FAD cofactor and Succinate substrate were docked into the models using Glide (Schrodinger), and the position of the ligands in available crystal structures used to guide placement. The quality of the models was confirmed with Verify3D (data not shown). Model structures were examined using Pymol. The model of the succinate complex was built using our previously reported models of SDHB and SDHD, with the X-ray crystal structure of the Avian respiratory complex ii (PDB ID: 1YQ4) (Huang et al. 2006) was used to guide protein docking.

The structural consequences of all the identified novel and previously identified SDHA missense variants were analyzed to account for all the potential effects of the mutations (Pires et al. 2016). The effects of the mutations upon the stability of SDHA were predicted using DUET (Pires et al. 2014a,b), an integrated computational approach that optimizes the prediction of two complementary methods (mCSM-Stability and SDM). The effect of the mutations upon the protein–protein binding affinity of SDHA to form the succinate complex were predicted using mCSM-PPi (Pires et al. 2014a; Pires and Ascher 2016). The effect of the mutations upon the binding affinity of SDHA for the cofactor, FAD, and substrate, succinate, were predicted using mCSM-Lig (Pires et al. 2015, 2016). These computational approaches represent the wild-type residues structural and chemical environment of a residue as a graph-based signature in order to determine the change upon mutation in Gibb’s free energy of stability or binding. To compare the in silico predictions for germline SDHA missense mutations detected in patients with those not ascertained via diagnostic testing, we identified 24 rare (frequency <0.01%) germline SDHA missense variants present in the ESP6500 cohort from Exome Variant Server (http://evs.gs.washington.edu) and correlated the effect of these missense variants on protein stability, complex formation, and ligand binding using our in silico prediction approaches. In addition, eight presumed missense somatic SDHA variants detected in SDH-related tumor types (seven renal cell carcinomas and one phaeochromocytoma) from the cBioPortal for cancer genomics (http://www.cbioportal.org), were evaluated.

Modeling of mammalian alignment to detect domains of purifying selection using SDHA transcript

Mammalian alignment from the 100 vertebrate genomes alignment for NM_004168.2 was downloaded via Table Browser at UCSC https://genome.ucsc.edu/cgi-bin/
Predicting the effects of the variants on splice regulatory information

See Appendix S7.

Statistical analysis

Statistical tests were performed using SPSS. Student’s t-test was used to compare continuous variables and Mann–Whitney or an unpaired t-test to compare nonnormally distributed data when sample numbers were small. Summary statistics included means and standard deviations for continuous variables, and frequencies and percentages for categorical variables.

Results

UK SDHA germline mutation series

Molecular genetics

Fifteen previously unreported patients with ten different germline SDHA variants were identified (Table 1). Two mutations had been reported previously: the common nonsense mutation c.91C>T (p.Arg31*) was observed in five patients and a c.1753C>T (p.Arg585Trp) missense mutation in one patient. A novel truncating mutation in c.1468G>T (p.Glu490*) was identified in one patient with a metastatic GIST tumor. Four further novel candidate missense variants, one frameshift variant and one splice acceptor variant were detected in six kindreds (see Table 1) (a novel missense variant, c.923C>T (p.Thr308Met) in exon 8 of SDHA was detected in two apparently unrelated patients).

Clinical features

Four patients presented with a GIST and eleven patients presented with a PCC/PGL. The mean age of disease presentation was 37.1 years (SD 14.2) with a range of 12–65 years. None of the affected individuals had a family history of SDH-related tumors. One proband with a truncating SDHA mutation (c.91C>T p.Arg31*) had a first degree relative tested after diagnosis who is an asymptomatic mutation carrier at age 72 years. One patient had died from another disease process at the time of this review. One patient with the c.923C>T (p.Thr308Met) missense variant was diagnosed with a malignant mediastinal paraganglioma at age 43 years and the second patient presented at a later age (52 years) with multiple bilateral HNPGL and a unilateral PCC. Further unreported variants included a missense variant in c.1273G>A (p.Val425Met) in a 62-year-old gentleman presenting with a para-spinal PGL and unilateral PC, two further missense mutations; c.133G>A (p.Ala45Thr) in exon 2 in a young male with a mediastinal PGL and c.136A>G (p.Lys46Glu) in exon 2, which was detected in a girl presenting of age 12 with a porta hepatis PGL. A novel truncating mutation [c.1468G>T (p.Glu490*)] was identified in a male patient, who presented aged 32 years with a GIST and later aged 36 and 38 years developed liver and lung metastases. The final two novel variants detected included a frameshift mutation (c.1338delA) in a 48-year-old female with a HNPGL and a splice mutation (c.1909-2A>G) in a 31-year-old female with a GIST.

Literature review of germline and somatic SDHA mutations

Germline SDHA mutations

Of 17 unique germline SDHA variants were identified in 47 individuals from 45 kindreds (Table S1). Three recurrent germline variants were identified: c.91C>T (p.Arg31*) nonsense variant in 22 kindreds (23 affected individuals) and two missense variants: c.1753C>T (p.Arg585Trp) (in two kindreds and two affected individuals) and c.1765C>T (p.Arg589Trp) (in four kindreds and four affected individuals). Details of clinical phenotype (Table S1) revealed that the most common association was with GIST tumors (mean age at diagnosis 33.4 years (SD+11.1), range 17–62 years) occurring in 31 of the 47 affected individuals. Five reported cases of metastatic GIST with SDHA germline variants were identified: two cases in association with a c.91C>T (p.Arg31*) (Pantaleo et al. 2011a,b; Italiano et al. 2012) and three further cases of metastatic GIST have been published in patients with the following mutations in SDHA: c.1151C>G (p.Ser384*) (Pantaleo et al. 2011a,b), c.1765C>T (p.Arg589Trp) (Wagner et al. 2013), and c.1534C>T (p.Arg512*) (Wagner et al. 2013) Reports of the PCC/PGL phenotype included eight PGL (abdominal or thorax), four HNPGL, and one PCC. There were
two reports of malignant PCC/PGL in association with germline SDHA variants. One patient with a sympathetic bladder PGL and a c.91C>T (p.Arg31*) variant (Burnichon et al. 2012) and a second patient with a HNPGL and a c.1534C>T (p.Arg512*) variant (Papathomas et al. 2015). No case of multifocal PCC/PGL was identified. One report of nonfunctioning pituitary macroadenoma and a germline SDHA variant was identified (Dwight et al. 2013a). Three recent case reports of renal cell carcinoma (RCC) in association with a SDHA variant have been published (Jiang et al. 2015; Ozluk et al. 2015; Yakirevich et al. 2015). One patient had a novel germline variant in c.2T>C (p.M1T) in the initiation codon of SDHA (Jiang et al. 2015) and was diagnosed with a renal cell chromophobe tumor and a multifocal GIST tumor. The two further reports were associated with somatic mutations and are described below.

An incomplete penetrance pattern with SDHA mutations is suggested by the sparse number of familial cases identified. Only two familial SDHA mutations were reported: two sisters with a c.91C>T (p.Arg31*) variant and GIST (Oudijk et al. 2013) and an additional family with a c.1873C>T (p.His625Tyr) variant, where the mother was the proband and had a HNPGL and her son had a nonfunctioning PA (Dwight et al. 2013b). The characteristics and population frequency of individual SDHA mutations described in the literature are described in Tables S1 and S2. The recurrent c.91C>T (p.Arg31*) nonsense mutation is recorded as occurring in 0.2 per 1000 individuals in the EXAC database (exac.broadinstitute.org/about) and all except one of the putative germline variants in our UK series and in the literature had a frequency of <1 per 1000 individuals in the EXAC dataset. However, a c.113A>T (p.Asp38Val) missense substitution described (Italiano et al. 2012) as a somatic mutation in a 26-year-old female with a metastatic GIST tumor with liver and peritoneal metastasis was present in 3.5% of individuals in the EXAC database.

A total of nine germline variants (three missense, six truncating) in SDHA, associated with either optic atrophy or Leigh syndrome were identified in the literature (Table S3). The only germline variant associated with both Leigh syndrome/optic atrophy and tumorigenesis including GIST and PCC/PGL was the c.91C>T (p.Arg31*) truncating variant.

Somatic SDHA mutations

Eleven cases of somatic candidate SDHA mutations were identified in the literature (Table S1): seven missense variants and four truncating. The associated tumor types included: GIST (n = 8), RCC (n = 2) and PA (n = 1). Two cases of RCC are associated with somatic SDHA variants (Ozluk et al. 2015; Yakirevich et al. 2015) and had histologic features, which were consistent with the histology typically associated with SDHB associated RCC (Ozluk et al. 2015; Yakirevich et al. 2015). One patient with a novel somatic 17 kbp SDHA homozygous deletion on chromosome 5p15, had malignant RCC (Yakirevich et al. 2015).

In silico structural analysis of germline and somatic SDHA variants associated with tumorigenesis

Computational approaches were employed to assess the effects of mutations on protomer stability, complex
formation and ligand binding to classify all identified SDHA missense variants associated with tumorigenesis in the literature and our unpublished cohort. A total of 18 putative missense mutations (11 germline and seven somatic) were analyzed. The data obtained from this in silico analysis were compiled with other predictive tools and a classification of these missense variants was made based on the ACMG recommendations (Richards et al. 2015) from the existing criteria available on each variant.

The mean DUET stability score was $-0.52$ kcal/mol (SD 0.936) for the 18 missense variants associated with tumorigenesis (mean $-0.53$ kcal/mol for 11 germline variants and $-0.48$ kcal/mol for seven somatic variants). The mean DUET score for missense variants ($n = 3$) reported in association with Leigh syndrome/optic atrophy was $-1.15$ kcal/mol.

The most destabilizing germline mutation predicted by DUET was $-1.81$ kcal/mol and associated with the missense variant c.1766G>A (p.Arg589Gln). This missense variant was detected in a single case of GIST in the literature. The second highest DUET score was associated with the germline missense variant c.1766C>T (p.Arg589Trp) which was identified in one patient in our study cohort with a GIST, and has been identified in the literature in one other patient with GIST and two patients with paragangliomas (see Table 3). Overall the most destabilizing mutation predicted by DUET was $-3.1$ kcal/mol and associated with a somatic mutation (c.1361C>A p.Ala454Glu) identified in a single case of GIST in the literature (see Table S1). Interestingly this variant was associated with loss of SDHB staining on immunohistochemistry but retained SDHA staining. This in silico prediction tool, predicted that the variant was highly destabilizing. It is in the FAD binding pocket and the mutation would abolish FAD binding and disrupt formation of the succinate complex.

The variant c.923C>T (p.Thr308Met) identified in two unrelated patients in our cohort with aggressive phenotypes (see Table 1) was predicted to mildly destabilize the protein protomer and part of substrate binding with a DUET score of $-0.498$ kcal/mol. No significant differences were detected between DUET scores of missense variants associated with GIST and with PCC/PGL ($P = 0.2$).

The in silico prediction tool predicted that 8/18 missense variants analyzed would have a mild or no effect on protein stability. Two of the eight variants were somatic variants; c.113A>T (p.Asp38Val) identified in a single GIST in the literature and the c.1334C>T (p.Ser445Leu) variant, also detected in a single GIST in the literature. The remaining six variants were germline and four of the six variants were identified in our novel UK cohort; c.133G>A (p.Ala45Thr), c.136A>G (p.Lys46Glu), c.923 C>T (p.Thr308Met), c. 1273G>A (p.Val425Met). A potential alternative mechanism for pathogenicity could be postulated for three N-terminal missense substitutions: c.113A>T (p.Asp38Val), c.133G>A (p.Ala45Thr) and c.136A>G (p.Lys46Glu), which were predicted to affect the transit peptide and potentially alter protein localization (see Table 3). One variant, c.1690G>A (p.Glu564Lys), was predicted to destabilize complex formation by mCSM-PPI (score of $-0.951$ kcal/mol).

A total of 8 somatic missense SDHA variants were identified from the cBioportal (http://www.cbioportal.org) in tumors associated with the SDHA disease phenotype. The mean DUET score in this group was $-0.94$ kcal/mol and 75% (6/8) of the missense variants were predicted to destabilize the protein or its ability to bind the substrate or form a complex (see Table S5). Three of these mutations were also predicted to affect complex formation (average mCSM-PPI score of $-1.025$ kcal/mol).

### In silico structural analysis of germline SDHA variants in control dataset

If SDHA pathogenic variants are usually associated with a low penetrance phenotype it might be postulated that rare pathogenic variants might also be detected in the general population. We therefore analyzed 24 rare (<0.05%) missense variants reported in a control data set (EVS6500, http://evs.gs.washington.edu) but not present in patient cohorts (Table S4) for predicted effect on protein stability, protein–protein and protein–ligand affinity and found that most 75% (18/24) were predicted to have a destabilizing effect and 41.6% (10/24) were predicted to affect complex formation. The in silico predictions of DUET correlated with SIFT and Polyphen prediction tools for 58.3% (14/24) of the variants. Additionally, 75% (6/8) of the somatic missense variants identified in RCC and 1 PCC tumor described in the CBioportal database were predicted to destabilize the protein (7/8) were predicted to be deleterious by SIFT/Polyphen (see Table S5).

### Tumor analysis in UK cohort

Two tumor specimens from our unpublished cohort were available for analysis. SDHA sequence analysis on a PGL from a patient with a c.1753 C>T variant (p.Arg585Trp) demonstrated partial loss of the wild-type allele in the tumor DNA consistent with pathogenicity. Tumor tissue from a patients with a c.91C>T (p.Arg31*) confirmed the presence of the variant but no loss of the wild type allele was detected (data not shown).

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**A Review of SDHA Variant Interpretation**

R.T. Casey et al.

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Classification of potential pathogenicity of germline SDHA variants associated with disease in our cohort and literature

Data from the in silico protein stability and affinity predictions was collated with data from computational predictive analyses and tumor studies in order to classify 18 identified missense variants as per the ACMG guidelines (Richards et al. 2015). 13/18 (72.2%) missense variants met the criteria for a pathogenic (or likely pathogenic) mutation (see Table 2). Five variants (29.5%) did not meet the criteria for a pathogenic mutation, and the supporting evidence was suggestive of a likely benign variant for four variants and one variant was classified as a variant of uncertain significance (VUS) because of insufficient evidence to classify as benign or pathogenic.

The five variants included four novel variants identified in our UK cohort and one variant identified from the literature. The first variant c.923C>T (p.Asp38Val), was a somatic variant and identified from the literature in a patient with a metastatic GIST. This variant was predicted to be benign by SIFT and Polyphen 2 prediction and is frequent in healthy controls (2.1%). It was predicted to cause potential disruption to the transit peptide on our in silico analysis tools and occurred at a frequency of up to 0.03% in healthy controls (see Table 1). These variants were predicted as benign variants across different computational analysis tools and occurred at a frequency of up to 0.03% in healthy controls (see Table 1). These variants were predicted as having a potential effect on the transit peptide but the DUET, mCSM-PPI and mCSM-Lig scores could not be calculated and there was no effect on the cofactor.

The remaining variants were suspected to be pathogenic. The first variant c.923C>T (p.Thr308Met), was detected in two patients who were not known to be related. This variant was associated with a malignant mediastinal PGL in one patient with a metastatic GIST and SDHA immunostaining was demonstrated in both tumor types. Taking a closer look at this mutation at a molecular level, however, reveals that His625 establishes an intricate network of polar interactions, including ionic interaction with Asp135 and Asp289, a donor–pi interaction with a Glu288 and a main-chain to main-chain hydrogen bond with Arg642 (depicted in Fig. 1). These would most likely be disrupted by the mutation to Tyr, destabilizing the protein.

The three remaining variants classified as likely benign and the variant classified as a VUS were identified in our novel cohort. One limiting factor to this classification was that tumor tissue was not available and so the variants could not be fully assessed. The first two variants c.133G>A (p.Ala45Thr) detected in a patient with a thoracic PGL and the c.136A>G (p.Lys46Glu), identified in a patient with an abdominal PGL, were consistently predicted as benign variants across different computational analysis tools and occurred at a frequency of up to 0.03% in healthy controls (see Table 1). These variants were predicted as having a potential effect on the transit peptide but the DUET, mCSM-PPI and mCSM-Lig scores could not be calculated and there was no effect on the cofactor.

The remaining variants were suspected to be pathogenic. The first variant c.923C>T (p.Thr308Met), was detected in two patients who were not known to be related. This variant was associated with a malignant mediastinal PGL in one patient with a metastatic GIST and SDHA immunostaining was demonstrated in both tumor types. Taking a closer look at this mutation at a molecular level, however, reveals that His625 establishes an intricate network of polar interactions, including ionic interaction with Asp135 and Asp289, a donor–pi interaction with a Glu288 and a main-chain to main-chain hydrogen bond with Arg642 (depicted in Fig. 1). These would most likely be disrupted by the mutation to Tyr, destabilizing the protein.

### Table 2. Classification of potential pathogenicity of SDHA missense variants identified in literature and novel UK cohort as per ACMG guidelines

<table>
<thead>
<tr>
<th>Variant</th>
<th>Effect</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.113A&gt;T (p.Asp38Val)</td>
<td>Likely benign (II)</td>
<td>PP5, PP4, BP1, BP4, BS1</td>
</tr>
<tr>
<td>c.133G&gt;A (p.Ala45Thr)</td>
<td>Likely benign (II)</td>
<td>PP4, BP1, BP4, PS3</td>
</tr>
<tr>
<td>c.136A&gt;G (p.Lys46Glu)</td>
<td>Likely benign (II)</td>
<td>PP4, BP1, BP4</td>
</tr>
<tr>
<td>c.511C&gt;T (p.Arg171Cys)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP4, BP1, PP3</td>
</tr>
<tr>
<td>c.562C&gt;T (p.Arg188Trp)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.767C&gt;T (p.Thr256Ile)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.800C&gt;T (p.Thr267Met)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.923C&gt;T (p.Thr308Met)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.1255G&gt;A (p.Gly419Arg)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PS3, BP1</td>
</tr>
<tr>
<td>c.1273G&gt;A (p.Val425Met)</td>
<td>Likely benign (II)</td>
<td>BP1, PP4, BP4</td>
</tr>
<tr>
<td>c.1334C&gt;T (p.Ser445Leu)</td>
<td>Likely pathogenic (III)</td>
<td>BP1, PP3, PS3, PP4</td>
</tr>
<tr>
<td>c.1361C&gt;A (p.Ala454Glu)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, PP5</td>
</tr>
<tr>
<td>c.1690G&gt;A (p.Glu564Lys)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP4, PP3, BP1</td>
</tr>
<tr>
<td>c.1753C&gt;T (p.Arg585Trp)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.1765C&gt;T (p.Arg589Trp)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP5, PP3, PP4, BP1</td>
</tr>
<tr>
<td>c.1766G&gt;A (p.Arg589Gln)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP4, PP5, PP3, BP1</td>
</tr>
<tr>
<td>c.1794G&gt;C (p.Lys598Asn)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, PP5, BP1</td>
</tr>
<tr>
<td>c.1873C&gt;T (p.His625Tyr)</td>
<td>Likely pathogenic (III)</td>
<td>PS3, PP3, PP4, PP5, BP1</td>
</tr>
</tbody>
</table>
Table 3. Structural Impact of 18 SDHA Missense substitutions on in silico protein models and correlation with other predictive tools.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Phenotype</th>
<th>DUET score (kcal/mol)</th>
<th>mCSM-PPI score (kcal/mol)</th>
<th>Effect on protein</th>
<th>Effect on co-factor</th>
<th>SIFT/Polyphen prediction</th>
<th>Heterozygous frequency per 1000 healthy population</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.113A&gt;T  (p.Asp38Val)</td>
<td>GIST</td>
<td>NA</td>
<td>NA</td>
<td>Transit peptide</td>
<td>No</td>
<td>Benign</td>
<td>21.7</td>
</tr>
<tr>
<td>c.133G&gt;A  (p.Ala45Thr)</td>
<td>Thoracic PGL</td>
<td>NA</td>
<td>NA</td>
<td>Near transit peptide</td>
<td>No</td>
<td>Benign</td>
<td>0.34</td>
</tr>
<tr>
<td>c.136A&gt;G  (p.Lys46Glu)</td>
<td>Abdominal PGL</td>
<td>NA</td>
<td>NA</td>
<td>Near transit peptide</td>
<td>No</td>
<td>Benign</td>
<td>0.24</td>
</tr>
<tr>
<td>c.511C&gt;T  (p.Arg171Cys)</td>
<td>GIST</td>
<td>-1.183</td>
<td>-0.592</td>
<td>Destabilizes protomer and complex</td>
<td>Yes</td>
<td>Damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.562C&gt;T  (p.Arg188Trp)</td>
<td>GIST</td>
<td>-0.901</td>
<td>-0.235</td>
<td>Destabilizes protomer</td>
<td>Yes</td>
<td>N/A</td>
<td>Not described</td>
</tr>
<tr>
<td>c.767C&gt;T  (p.Thr256Ile)</td>
<td>GIST</td>
<td>-0.397</td>
<td>-0.397</td>
<td>Destabilizes protomer and complex</td>
<td>Yes</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.800C&gt;T  (p.Thr267Met)</td>
<td>GIST</td>
<td>0.77</td>
<td>-0.287</td>
<td>Substrate binding pocket</td>
<td>Yes</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.923C&gt;T  (p.Thr308Met)</td>
<td>HNPGL, Thoracic PGL</td>
<td>-0.498</td>
<td>-0.16</td>
<td>Mildly destabilizes protomer and part of substrate binding site</td>
<td>No</td>
<td>Benign</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1255G&gt;A (p.Gly419Arg)</td>
<td>GIST</td>
<td>-1.268</td>
<td>0</td>
<td>Destabilizes protomer</td>
<td>No</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1273G&gt;A (p.Val425Met)</td>
<td>PGL</td>
<td>0.083</td>
<td>0</td>
<td>No effect</td>
<td>No</td>
<td>Probably damaging</td>
<td>0.02</td>
</tr>
<tr>
<td>c.1334C&gt;T (p.Ser445Leu)</td>
<td>GIST</td>
<td>0.971</td>
<td>0</td>
<td>Stabilizes protomer</td>
<td>No</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1361C&gt;A (p.Ala454Glu)</td>
<td>GIST</td>
<td>-3.1</td>
<td>-1.65</td>
<td>Destabilizes complex</td>
<td>Yes</td>
<td>Damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1690G&gt;A (p.Glu564Lys)</td>
<td>GIST</td>
<td>0.263</td>
<td>-0.951</td>
<td>Destabilizes complex</td>
<td>Yes</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1753C&gt;T (p.Arg585Trp)</td>
<td>PGL, PC</td>
<td>-1.09</td>
<td>0</td>
<td>Destabilizes protomer</td>
<td>No</td>
<td>Damaging</td>
<td>0.002</td>
</tr>
<tr>
<td>c.1765C&gt;T (p.Arg589Trp)</td>
<td>GIST, PGL</td>
<td>-1.383</td>
<td>0</td>
<td>Destabilizes protomer</td>
<td>No</td>
<td>Damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1766G&gt;A (p.Arg589Gln)</td>
<td>GIST</td>
<td>-1.81</td>
<td>0</td>
<td>Destabilizes protomer</td>
<td>No</td>
<td>Probably damaging</td>
<td>Not described</td>
</tr>
<tr>
<td>c.1794G&gt;C (p.Lys598Asn)</td>
<td>GIST</td>
<td>0.301</td>
<td>0</td>
<td>No effect</td>
<td>No</td>
<td>N/A</td>
<td>0.016</td>
</tr>
<tr>
<td>c.1873C&gt;T (p.His625Tyr)</td>
<td>1 PA, 1 HNPGL</td>
<td>0.059</td>
<td>0</td>
<td>No effect</td>
<td>No</td>
<td>N/A</td>
<td>Not described</td>
</tr>
</tbody>
</table>
patient and multiple PGL and a PCC in a second patient. This variant was predicted to be benign by SIFT and PolyPhen but has not been identified in healthy controls and was also found to mildly destabilize the protomer and substrate binding site and therefore is likely to affect protein stability. Thr308 establishes, apart from hydrophobic interactions, hydrogen bonds that would be lost by the substitution to Methionine, which could also induce steric clashes (Fig. 2). Its proximity to the ligand FAD, could also imply a change in substrate binding as well. However due to insufficient evidence, the default classification of this variant was VUS. The final variant c.1273G>A (p.Val425Met), was detected in a patient with a spinal PGL and PCC. This variant was predicted to be pathogenic by SIFT and PolyPhen and is only present in .002% of healthy controls. However this variant was not found to impact on protein stability by our in silico prediction analysis. As functional studies were not performed on this variant the overall criteria for a likely pathogenic variant were not met and the classification was a likely benign variant as per ACMG (see Table 2).

**Prediction of splice disruption**

Appendix S7.

**Variants plotted to domains of strong purifying selection on mammalian alignment**

A priori we might expect disease causing mutations to be more common in domains of high-conservation within a gene, although disruption of such domains can also result in early embryonic mortality and so not be considered pathogenic. Calculation of SDHA Ka/Ks ratios for human versus marmoset (*Callithrix jaccus, callJac3 assembly*) and bushbaby (*Otelemur garnetti otoGar3 assembly*) revealed evidence for domains of strong purifying selection (Ka/Ks < 0.1) across multiple spans of the gene (Fig. 3). A total of 29.4% (*n* = 5/17) of the analyzed SDHA missense variants mapped to domains of strong purifying selection and 46% (*n* = 11/24) missense variants identified in healthy controls (see Table S6) (frequency <0.01%) (from Exome Variant Server (http://evs.gs.washington.edu). These frequencies are not significantly different (chi squared = 0.21, *P* = 0.88).

**Discussion**

To date, germline mutation analysis of SDHA has not been widely adopted in clinical practice. In part this relates to the more recent (compared to SDHB/C/D)
association of SDHA mutations with tumorigenesis (Pantaleo et al. 2011a, 2011b; Burnichon et al. 2010; Dwight et al. 2013b; Jiang et al. 2015), but also to technical challenges (molecular genetic analysis of SDHA is complicated by its four known pseudogenes, generated by complete or partial gene duplications (Rattenberry et al. 2013).

Although the SDHA/B/C/D subunits form a single complex, mutations in different genes are associated with relative differences in susceptibility to specific tumor types. Thus, whilst SDHA is often not tested routinely for in PCC/PGL/HNPGL cases, it does appear to be a much rarer cause of PCC/PGL/HNPGL than SDHB and SDHD mutations. However, though other mutations in other SDH subunit genes may also be associated with GIST, the relative frequency of SDHA mutations reported in association with GIST appears much higher than other subunits (Boikos et al. 2016). Interestingly, SDHA-associated GIST, has been reported to occur at an older age and have less female preponderance (Miettinen and Lasota 2014). Nevertheless, the tumorigenic effects of SDHA mutations are thought to be mediated through similar mechanisms as for mutations in other SDH subunits e.g. through a pseudohypoxic drive, facilitating angiogenesis and aberrant cell proliferation (López-Jiménez et al. 2010) and epigenetic effects through the accumulation of succinate, and subsequent inhibition of demethylase enzymes resulting in promoter hypermethylation and tumor suppressor gene inactivation (Letouzé et al. 2013).

Since SDHA mutations were initially associated with PCC/PGL the spectrum of associated tumors has expanded to also include HNPGL, GIST, renal tumors, and pituitary adenoma (PA) (Pantaleo et al. 2011a, 2011b; Burnichon et al. 2010; Dwight et al. 2013b; Jiang et al. 2015). Thus the detection of a rare putative SDHA mutation might have clinical significance. However, SDHA mutations appear to have reduced penetrance (multiple affected individuals within a single family are rare) and SDHA mutations (e.g. c.91C>T p.Arg31*) can occur in healthy individuals at a population frequency of between 1/1000 and 1/10,000 (see Table 2). Thus the interpretation of the contribution of a putative novel germline SDHA mutation to the observed phenotype may not be straightforward as familial segregation studies are unlikely to be informative and the presence of the variant in control populations does not exclude pathogenicity. Interestingly a high variant density has been identified for SDHA in African American samples (Baysal et al. 2007). This increased variant expression was initially attributed to higher rates of gene recombination, however a study using a high resolution recombination map disputed this theory as a low recombination rate at the locus of the SDHA gene was observed (Myers et al. 2005). It is now considered more likely that the four known SDHA pseudogenes have contributed to increased SDHA variant density by illegitimate recombination or gene conversion at the time of meiosis.

Bioinformatic prediction tools such as Polyphen and SIFT are widely used to aid the interpretation of the likely pathogenicity of sequence variants, although it is well recognized that they have their limitations. Previously, we and others have found that in silico structural prediction analysis tools can aid the classification of germline SDHB and SDHD variants (Ricketts et al. 2010). Although we found that most putative SDHA mutations detected in patients presenting with a relevant tumor were reported to impair protein stability, we also found that many rare SDHA missense variants present in the ESP6500 exome sequencing data set were also predicted to be destabilizing by DUYET and pathogenic by PolyPhen/SIFT. Though no information is available on the phenotype of ESP6500 individuals with SDHA variants, this comparison does illustrate the challenge in interpreting the significance of rare genetic variants in candidate genes.

Identification of rare genetic variants associated with inherited tumor predisposition can enable testing of at risk relatives (and appropriate surveillance of mutation carriers), enhanced surveillance (if they are at increased risk of second primary tumors) and, if applicable, targeted therapy for the affected individual. In the case of putative SDHA mutations, the evidence for incomplete penetrance and lack of information on tumor risks in non-probands suggests that the genetic testing and intensive surveillance of at risk family members will generally not be indicated until more information on the genetic epidemiology and age-related tumor risks are available. For affected individuals with putative missense mutations, we suggest that, in addition to in silico protein structure and bioinformatic predictions of pathogenicity (e.g. SIFT/ PolyPhen), additional studies should be undertaken to aid variant classification. Loss of heterozygosity (LOH) analysis of tumors can support a case for pathogenicity if there is loss of the wild type allele. Though the absence of LOH does not exclude pathogenicity of the variant as other mechanisms such as somatic point mutations or promoter hypermethylation can inactivate the wild-type allele without causing LOH (as seen in our case with a c.91 C>T (p.Arg31*) variant and reported by others (Lussey-Lepoutre et al. 2015). Tumor immunohistochemistry (IHC) can also support pathogenicity by demonstrating the loss of SDHA expression (Miettinen et al. 2013). However discrepancies between IHC results and predicted pathogenicity of SDH gene variants, appear to be more common for SDHA variants identified in patients with GIST (Evenepoel et al. 2015).

Furthermore, we suggest that there should also be an increased emphasis on defining whether a rare germline
SDHA variant is associated with the expected functional consequences of SDHA inactivation in the relevant tumor. Thus metabolomic analysis using in vivo MRI spectroscopy (MRS) or in vitro high resolution magic angle spinning (HRMAS), have recently been reported as useful diagnostic adjunct in patients with putative SDHX gene mutations. Peaks in the metabolite succinate in tumor tissue as a result of a defective succinate dehydrogenase enzyme, have been demonstrated as a sensitive and specific hallmark of SDH mutations (Imperiale et al. 2015; Lussey-Lepoutre et al. 2015) and have been described in an abdominal PGL associated with a germline SDHA c.91C>T (p.Arg31*) mutation (Lussey-Lepoutre et al. 2015). Similarly, methylome profiling can be used to identify the hypermethylation epigenetic alterations associated with SDHx inactivation (Letouzé et al. 2013). The correct classification of putative SDHA mutations and the demonstration of the expected abnormal tumor metabolic/epigenetic profile will become increasingly important as targeted therapies based on derangements in the metabolic/epigenetic abnormalities are developed and studied.

In conclusion, this review of published SDHA mutations and reporting of variants from our novel cohort, should aid interpretation of genetic testing results in patients with relevant tumor types. We advise that caution should be exercised in interpreting pathogenicity of novel rare sequence variants and that, in such cases, whenever possible a variety of strategies, including structural prediction analysis and molecular genetics, SDHB/SDHA immunohistochemical analysis, metabolomic and methylome profiling of tumors should be performed, to better define the likelihood of pathogenicity of SDHA variants to ensure optimum clinical management.

Conflict of Interest

None of the authors have anything to declare. This research has not been presented at any meeting to date.

References

A Review of SDHA Variant Interpretation


Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Methods and Results.
Table S1. SDHA variants (both germline and somatic) described in literature.
Table S2. Characteristics of pathogenic variants reported in the literature.
Table S3. Variants associated with optic atrophy or Leigh syndrome.
Table S4. SDHA variants identified from EVS not associated with disease in our cohort or Literature.
Table S5. SDHA variants identified as somatic mutations in related tumor types in CBioportal.
Table S6. Variants that mapped to domains of strong purifying selection on mammalian alignment.
Table S7. Predicted effects of mutations on splicing. To determine the effect of exonic mutations on splicing we considered both whether they were at splice sites (distance = 0 – light green highlight) and whether they were predicted to have a significant effect on the density of exonic splice enhancers and suppressors. The change in exonic splice regulation score is given in column 3, with a Z score and P value (from simulation) in columns 4 and 5. A negative Z score is considered as a prediction of disrupted splicing. Mutations predicted to disrupt exonic splice enhancer motifs at $P < 0.05$ are shown in yellow.
Clinical and Molecular Features of Renal and Pheochromocytoma/Paraganglioma Tumor Association Syndrome (RAPTAS): Case Series and Literature Review

Ruth T. Casey,1,2 Anne Y. Warren,3 Jose Ezequiel Martin,1 Benjamin G. Challis,2 Eleanor Rattenberry,4,5 James Whitworth,1 Katrina A. Andrews,1 Thomas Roberts,6 Graeme R. Clark,1 Hannah West,1 Philip S. Smith,1 France M. Docquier,1 Fay Rodger,1 Vicki Murray,1 Helen L. Simpson,4 Yvonne Wallis,4 Olivier Giger,3 Maxine Tran,7 Susan Tomkins,8 Grant D. Stewart,9 Soo-Mi Park,1 Emma R. Woodward,5 and Eamonn R. Maher1

1Department of Medical Genetics, University of Cambridge and National Institute for Health Research Cambridge Biomedical Research Centre and Cancer Research UK Cambridge Centre, Cambridge CB2 0QQ, United Kingdom; 2Department of Endocrinology, Cambridge University National Health Service (NHS) Foundation Trust, Cambridge CB2 OQQ, United Kingdom; 3Department of Histopathology, Cambridge University NHS Foundation Trust and Cancer Research UK Cambridge Centre, Cambridge CB2 OQQ, United Kingdom; 4West Midland Regional Genetics Laboratory, Birmingham Women’s NHS Foundation Trust, Birmingham B15 2TG, United Kingdom; 5Manchester Centre for Genomic Medicine, St Mary’s Hospital, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester M13 9WL, United Kingdom; 6Haematology Oncology Diagnostic Service, Cambridge University NHS Foundation Trust, Cambridge CB2 OQQ, United Kingdom; 7Division of Surgery and Interventional Science, University College London, Royal Free Hospital, London NW1 2BU, United Kingdom; 8Department of Clinical Genetics, University Hospitals Bristol NHS Foundation Trust, Bristol BS2 8HW, United Kingdom; and 9Academic Urology Group, University of Cambridge and Cancer Research UK Cambridge Centre, Addenbrooke’s Hospital, Cambridge CB2 OQQ, United Kingdom

Context: The co-occurrence of pheochromocytoma (PC) and renal tumors was linked to the inherited familial cancer syndrome von Hippel-Lindau (VHL) disease more than six decades ago. Subsequently, other shared genetic causes of predisposition to renal tumors and to PC, paraganglioma (PGL), or head and neck paraganglioma (HNPGL) have been described, but case series of non–VHL-related cases of renal tumor and pheochromocytoma/paraganglioma tumor association syndrome (RAPTAS) are rare.

Objective: To determine the clinical and molecular features of non-VHL RAPTAS by literature review and characterization of a case series.

Design: A review of the literature was performed and a retrospective study of referrals for investigation of genetic causes of RAPTAS.

Results: Literature review revealed evidence of an association, in addition to VHL disease, between germline mutations in SDHB, SDHC, SDHD, TMEM127, and MAX genes and RAPTAS [defined here as the co-occurrence of tumors from both classes (PC/PGL/HNPGL and renal tumors) in the same individual or in first-degree relatives]. In both the literature review and our case series of 22 probands with non-VHL RAPTAS, SDHB mutations were the most frequent cause of non-VHL RAPTAS. A genetic cause was identified in 36.3% (8/22) of kindreds.

Abbreviations: FDR, first-degree relative; HNPGL, head and neck paraganglioma; NGS, next-generation sequencing; PC, pheochromocytoma; PGL, paraganglioma; RAPTAS, pheochromocytoma/paraganglioma tumor association syndrome; RCC, renal cell carcinoma; SD, standard deviation; VHL, von Hippel-Lindau.
C

auses for the occurrence of different tumor types in the same individual or in close relatives may include shared environmental exposures and /or inherited neoplasia disorders. Combinations of specific tumor types may strongly implicate specific inherited cancer syndromes (1). Thus the combination of pheochromocytoma (PC) and renal cell carcinoma (RCC) was recognized as a “form fruste” of von Hippel-Lindau (VHL) disease more than 60 years ago (2). RCC is the most common form of adult renal cancer, and ~3% occurs from a hereditary disorder (3). PC and paraganglioma (PGL) are functional neuroendocrine tumors arising from the adrenal medulla (PC) or sympathetic ganglia (PGL) with an annual incidence of 2 to 8 per 1 million persons (4). The proportion of PC/PGL cases attributable to a genetic cause is at least 10-fold higher than for RCC (5); some genetic causes of PC/PGL also predispose to head and neck paraganglioma (HNPG). Nevertheless, the combination of RCC and PC/PGL in a single individual or close relatives is rare and, if cases of VHL disease are excluded, clinical and molecular studies are limited mostly to anecdotal case reports (6–9).

In this study, we have investigated the genetic architecture of the clinical association (in the same individual or family) of a renal tumor and a PC/PGL/HNPG without evidence of VHL disease [referred to here as non-VHL renal and pheochromocytoma/paraganglioma tumor association syndrome (RAPTAS)]. We undertook a comprehensive literature review and a retrospective study of a large case series of 22 probands (index cases) and 11 affected first-degree relatives (FDRs) referred to tertiary genetic services.

**Conclusion:** Renal tumors and PC/PGL/HNPG tumors share common molecular features and their co-occurrence in an individual or family should prompt genetic investigations. We report a case of MAX-associated renal cell carcinoma and confirm the role of TMEM127 mutations with renal cell carcinoma predisposition. *(J Clin Endocrinol Metab 102: 4013–4022, 2017)*

**Methods**

**Case series**

Details of patients referred for molecular genetic testing because of a suspected hereditary cause of PC/PGL or RCC over a period of 15 years (2001 through 2016) were reviewed and those with clinical (e.g., in addition to PC/RCC, the presence of retinal or central nervous system hemangio-blastoma, multiple renal or pancreatic cysts, pancreatic neuroendocrine tumors, endolymphatic sac tumors) or molecular evidence of VHL disease were excluded. Patients included had either (1) a personal history of PC/PGL/HNPG and a renal tumor or (2) the presence of PC/PGL/HNPG and RCC in FDRs (e.g., PC in a proband and RCC in a parent). Patients meeting these criteria were classified as having non-VHL RAPTA. Referral data from three UK National Health Service molecular diagnostic laboratories undertaking genetic testing were collated on a standardized pro forma and included sex, age at presentation, method of presentation (sporadic vs familial), location of tumor, presence of bilateral/multifocal disease, and evidence of malignancy. Molecular genetic testing information was also collected. Patients gave written informed consent to a research ethics committee–approved research study and/or data were collected as part of a molecular genetics service evaluation study.

**Molecular genetic testing of patients in case series**

Some cases referred before 2011 had individual gene testing (e.g., VHL, SDHB) but more recent cases were tested for a panel of up to 10 susceptibility genes (SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, MAX, TMEM127, RET, FH), mostly using a next-generation sequencing (NGS)-based assay described previously (10). All participants gave informed consent for clinical diagnostic genetic testing. NGS was performed using the Illumina or Ion Torrent platforms. On average, coverage depth of >20-fold was achieved for 98% of the regions sequenced. All pathogenic variants were confirmed by Sanger sequencing. Copy number changes in VHL, SDHB, SDHC, and SDHD were sought by multiple ligation probe analysis. Targeted tumor sequencing was performed on DNA extracted from four macro-dissected formalin-fixed paraffin-embedded tumor samples with a custom panel based on the Ion AmpliSeq™ Cancer Hotspot Panel v2 with additional bespoke content (Supplemental Table 4). The Covaris Adaptive Focused Acoustics™–based DNA extraction and purification from formalin-fixed paraffin-embedded tissue protocol was used and 20 ng of extracted DNA was sequenced. Library preparation was performed using an adapted Ampliseq on MiSeq or MiSeq2 primer protocol. Sequencing was performed on the Illumina MiSeq system.

**Bioinformatics and histology review**

See the Supplemental data for more information.

**Literature review**

A full review of the published literature on the genes reported to predispose to PC/PGL or RCC up to December 2016 was performed. This search was performed and included publications indexed in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) up to June 2017. Search terms included NFI, RET, MAX, EGLN1, EGLN2, MSH2, KIF1B, SDHAF2, MEN1, BAP1, CDC73, CDKN2B, FLCN, MET, PBRM1, PTEN, TSC1, TSC2, FH, SDHA, SDHB, SDHC, SDHD, TMEM127, and VH genes, hereditary, renal cell carcinoma, oncocytoma, kidney cancer, pheochromocytoma, and paraganglioma. In addition, the Humane Gene Mutation Database (www.hgmd.cf.ac.uk) and the Leiden Open Variation Database (http://www.lovd.nl/3.0/home) were reviewed. The search results were interrogated to identify genetic causes of RAPTA.

**MTS**

We applied the previously described multiple primary tumor score (MTS) (11) to group A.
Statistical analysis

Statistical tests were performed using SPSS. Summary statistics include mean and standard deviation (SD) for continuous variables and frequency and percentage for categorical variables. A two-sample t test was applied to parametric means and a Mann-Whitney test was applied as the nonparametric equivalent test. Fisher’s exact test was used to calculate the statistical difference between proportions of wild-type versus alternate allele reads.

Results

Case series demographics

Thirty-three individuals (16 males, 17 females) with PC/PGL/HNPGL and/or a renal tumor from 22 kindreds without clinical or molecular evidence of VHL disease met our criteria for the diagnosis of non-VHL RAPTAS. This cohort was subdivided into two groups: multiple tumor patients with a combination of PC/PGL/HNPGL + RCC (n = 12 probands; group A) and familial non-VHL RAPTAS cases with RCC or PC/PGL/HNPGL and an FDR with the alternative tumor type (n = 21 patients, 10 probands; group B).

Clinical features of group A: multiple tumor non-VHL RAPTAS cases

Twelve patients with a diagnosis of PC/PGL and a renal tumor were identified. The clinical details are summarized in Table 1. Seven cases had synchronous tumors and five metachronous. Mean age at diagnosis of first tumor was 55.3 years (SD, 19.4; range, 10 to 76 years). Four of five metachronous cases presented with PC/PGL/HNPGL and one patient was initially diagnosed with RCC. In most cases, a unilateral PC was present (75%, 9/12 patients), but there were two cases (16.6%) with HNPGL and one with an abdominal PGL. Most renal tumors were RCC (91.7%, 11/12 patients), but a renal oncocytoma was present in a patient without a

Table 1. Clinical Features and Genetic Features of RAPTAS Patients With Multiple Tumors

<table>
<thead>
<tr>
<th>Proband</th>
<th>Age (in Years)</th>
<th>Phenotype</th>
<th>Metastatic Disease</th>
<th>Germline Genetic Analysis</th>
<th>Histology Reviewed</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>at Diagnosis of First Tumor (Second Tumor)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>63 (63)</td>
<td>Unilateral renal oncocytoma</td>
<td>No</td>
<td>No detectable mutation in SDHA, SDHBI/SDHCSI,SDHD,SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>76 (76)</td>
<td>Unilateral RCC</td>
<td>No</td>
<td>No detectable mutation in SDHA, SDHBI/SDHCSI,SDHD,SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>56 (56)</td>
<td>Unilateral RCC</td>
<td>No</td>
<td>No detectable mutation in SDHA, SDHBI/SDHCSI,SDHD,SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>62 (64)</td>
<td>Unilateral PC</td>
<td>Yes (RCC)</td>
<td>No detectable mutation in SDHA, SDHBI/SDHCSI,SDHD,SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>68 (68)</td>
<td>Unilateral PC</td>
<td>No</td>
<td>No detectable mutation in SDHB or VHL</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>41 (41)</td>
<td>Carotid body PGL</td>
<td>No</td>
<td>Variant of uncertain significance SDHD (c.34G&gt;A p.Gly12Ser)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unilateral RCC</td>
<td></td>
<td>Tested for SDHB/C/D and VHL</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>60 (60)</td>
<td>Unilateral RCC</td>
<td>No</td>
<td>No detectable mutation in SDHB or VHL</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>10 (26)</td>
<td>Abdominal PGL</td>
<td>No</td>
<td>SDHB mutation c.141G&gt;A (p.TRp47*)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unilateral RCC</td>
<td></td>
<td>Tested for VHL and SDHB</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>62 (63)</td>
<td>Unilateral PC</td>
<td>No</td>
<td>SDHB mutation c.268C&gt;T (p.Arg90*)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unilateral RCC</td>
<td></td>
<td>Tested for SDHB, VHL</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>43 (43)</td>
<td>Unilateral RCC</td>
<td>No</td>
<td>MAX mutation c.97C&gt;T (p.Arg33*)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unilateral PC</td>
<td></td>
<td>Tested for SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, and FH</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>53 (62)</td>
<td>Unilateral PCC</td>
<td>No</td>
<td>TMEM127 mutation c.117_120delGCT (tested for SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, and FH)</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>34 (39)</td>
<td>Carotid body HNPGL</td>
<td>Yes (RCC)</td>
<td>SDHB mutation c.79C&gt;T (p.Arg27*), Tested for SDHB, SDHC, SDHD, and VHL</td>
<td>Yes</td>
</tr>
</tbody>
</table>
germline mutation. One group A patient had been diagnosed with breast carcinoma, but no additional tumors such as gastrointestinal stromal, thyroid, or pituitary tumors were identified in group A or group B patients (Tables 1 and 2).

**Clinical features of group B: familial non-VHL RAPTAS cases**

Ten kindreds were identified containing two or more FDRs with PC/PGL/HNPGL and a renal tumor. Information including phenotype, genotype, and demographic information was available on 10 probands (6 females, 4 males) referred for genetic testing and basic demographic/phenotype information was available on the 11 affected FDRs (2 females, 9 males) (Table 2). Mean age at presentation of the probands was 56.6 years (SD, 17.3; range, 27 to 77 years) and mean age at tumor diagnosis in 11 affected FDRs was 52.3 years (SD, 16.3; range, 13 to 65 years). In eight kindreds, the proband presented with a PC/PGL/HNPGL (three with a PC, three with HNPGL, and two with abdominal PGL) and in two cases had malignant PGLs (an HNPGL and an abdominal PGL) (Table 2). Two probands presented with RCC and a renal oncocytoma. Most probands in group B had one affected relative, but one proband had two affected relatives (two brothers, both with RCC).

**Molecular genetics analysis of the non-VHL RAPTAS case series**

Molecular genetic analysis was performed on all 22 probands from groups A and B. All cases were tested for germline mutations in VHL, and SDHB and 8/12 (67%) of probands from group A and 6/10 (60%) of probands from cohort B were also tested for mutations in SDHA, SDHC, SDHD, SDHAF2, FH, MAX, and TMEM127. A germline SDHB mutation (four truncating mutations and a splice site mutation) was detected in 6/22 (27.3%) probands (three from group A and three from group B). Family testing was possible in two of three group B kindreds; in both cases, the affected relative harbored the SDHB mutation detected in the proband.

One proband was diagnosed with a variant in SDHD (c.34G>A, p.Gly12Ser) that was not considered pathogenic and did not prompt family screening. One proband presenting with RCC and unilateral PC age 43 years had a truncating mutation in the MAX gene (Table 1). This NGS result was confirmed by Sanger sequencing. Another proband from group A was found to have a truncating mutation in TMEM127 (Table 1).

No statistically significant correlation was identified for younger age at first tumor diagnosis, PGL, renal oncocytoma or malignant PGL, and the identification of a genetic mutation (P > 0.05 for all associations). The mean MTS (11) value in group A patients with a mutation was 3.6 compared with 1.8 in those without a mutation (P = 0.09).

**Histology review**

Archival tumor samples were available for four patients from group A (RCC samples from probands 2, 3, 11, and 12 and a PC from proband 2) and histology review and SDHB immunostaining was performed (Figs. 1 and 2).

**Tumor sequencing**

Analysis of DNA extracted from the PC and RCC tumors from case 10 with the germline mutation in the MAX gene (c.97C>T p. Arg33*) revealed loss of heterozygosity (Supplemental Fig. 4), with higher reads in the mutant allele identified in the PC [reads wild-type/mutant: 77/151 (depth 228) and RCC (reads: 60/179, depth 239) compared with the germline (157/157, depth 239) compared with the germline (157/157, depth

<table>
<thead>
<tr>
<th>Proband No.</th>
<th>Age in Years at Diagnosis</th>
<th>Phenotype of Proband</th>
<th>Genetic Mutation Identified in Proband</th>
<th>Relative Affected</th>
<th>Phenotype of Relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>56</td>
<td>Renal oncocytoma</td>
<td>SDHB splice site intron mutation IVS1 + 1 G&gt;T</td>
<td>Daughter (13)</td>
<td>Unilateral PC</td>
</tr>
<tr>
<td>14</td>
<td>50</td>
<td>HNPGL</td>
<td>No detectable mutation in SDHB or VHL</td>
<td>Father (58)</td>
<td>Unilateral PC</td>
</tr>
<tr>
<td>15</td>
<td>77</td>
<td>Unilateral PC</td>
<td>No detectable mutation in SDHA, SDHBD/SDHD,SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>Daughter (51)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
<td>HNPGL</td>
<td>No detectable mutation in SDHA, SDHBD/SDHD,SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>Brother (54)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>17</td>
<td>57</td>
<td>Abdominal PGL</td>
<td>SDHB mutation c.166-170delCCTCA (p.Pro56Tyrfs5X)</td>
<td>Brother (57)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>18</td>
<td>67</td>
<td>Abdominal PGL</td>
<td>No detectable mutation in SDHB or VHL</td>
<td>Brother (52)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Unilateral PC</td>
<td>No detectable mutation in SDHA, SDHB, SDHC, SDHSD/SDHAF2, MAX, TMEM127, FH, VHL</td>
<td>Brother (65)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>Unilateral RCC</td>
<td>SDHB mutation c.380T&gt;G (p.Ile127Ser)</td>
<td>Brother (64)</td>
<td>Unilateral PC</td>
</tr>
<tr>
<td>21</td>
<td>60</td>
<td>Unilateral PC</td>
<td>No detectable mutation in SDHB or VHL</td>
<td>2 brothers (50,63)</td>
<td>Unilateral RCC</td>
</tr>
<tr>
<td>22</td>
<td>27</td>
<td>Unilateral PC</td>
<td>No detectable mutation in SDHB or VHL</td>
<td>Father (49)</td>
<td>Unilateral RCC</td>
</tr>
</tbody>
</table>

*Metastatic disease.
314) [germline.v’s.PC P = 0.0002; germline.v’s.RCC P < 0.0001 (Fisher’s exact test)]. No additional somatic homozygous mutations were identified in other RAPTAS-related genes (VHL, SDHA, SDHB, SDHC, SDHD, FH, TMEM127) in either tumor from case 10 (Supplemental Table 4).

Loss of SDHB immunostaining in the PC from case 2 prompted additional sequencing of tumor tissue from the PC and RCC because germline testing did not reveal a germline mutation in SDHx or VHL (Table 1). No somatic mutation in SDHA/SDHB/SDHC/SDHD was identified in either tumor, but a somatic variant (not present in the germline) in VHL (c.245G>T p Arg82Leu) was identified in the PC tumor but not the RCC from case 2.

Literature review

Germline mutations in at least 25 different genes have been reported to predispose to PC/PGL/HNPGL or RCC (NF1, RET, MAX, EGLN1, EGLN2, MSH2, KIFIB, SDHAF2, MEN1, BAP1, CDC73, CDKN2B, FLCN, MET, PBRM1, PTEN, TSC1, TSC2, FH, SDHA, SDHB, SDHC, SDHD, TMEM127, and VHL) (6–9, 12–14, 15). However, with the exception of VHL disease and, to a lesser extent, SDHB mutations (16–20), other reported genetic causes of RAPTAS (as defined here) are rare.

A total of 21 kindreds and 39 patients were identified from the literature with a non-VHL RAPTAS phenotype and a germline mutation. Mean age of tumor onset was 36.1 years (17 to 61 years) [31.8 years (17 to 47 years) for PC/PGL/HNPGL and 41.4 (19 to 61 years) years for PC/PGL/HNPGL and 41.4 (19 to 61 years) years for

Figure 1. (a) The hematoxylin and eosin (H+E)-stained compact architecture and overall histological features consistent with a clear-cell RCC from proband 11 with a TMEM127 mutation. (b) Positive SDHB immunostaining in the same RCC tumor from proband 11. (c) Histological examination of a chromophobe RCC tumor from proband 3 with no detectable germline mutation (H+E staining ×200 high-power field). There is evidence of pleomorphic nuclei and perinuclear halos. (d) Positive SDHB immunostaining of the chromophobe RCC tumor.

Figure 2. (a) The H+E-stained histological appearance of the SDHB-deficient RCC from proband 12. There is evidence of intracytoplasmic vacuoles marked by the black arrow. (b) Loss of SDHB protein expression on immunostaining of the RCC tumor from proband 12 in the lower part of the image, with SDHB staining present in the adjacent normal renal tissue visible in the upper image. (c) The histological appearances of a renal papillary carcinoma from proband 2 (H+E staining ×200 high-power field) and (d) preserved SDHB expression on immunostaining in this tumor. (e) A PC tumor from proband 2. (f) Negative SDHB immunostaining in the PC. The black arrow points to an area of normal adrenal tissue with preserved SDHB protein expression.
RCC). The most commonly mutated gene was SDHB (16/21 kindreds) and 44% (7/16) had a deletion (missegment in 5/16 and truncating mutations in 4/16). Most reported cases were diagnosed with an RCC. Metastatic RCC was reported in five patients with SDHB mutations, one patient with an SDHC mutation, and one patient with an SDHD mutation. Three cases had bilateral RCC and one bilateral PC. Metastatic PC/PGL/HNPGL occurred in one patient. Renal oncocytoma was described as part of three cases of RAPTAS (two with an SDHB mutation and one with a MAX mutation) (Table 3).

In addition to patients with RAPTAS, separate case reports of PC/PGL/HNPGL or renal tumors have been reported in association with the six genes described in Table 3, as well as with mutations in FH (14, 21) and SDHA (9, 22) (although no cases of coexisting PC/PGL and RCC in the same patient had been reported in conjunction with a mutation in FH/SDHA). Although there are very rare cases of tuberosesclerosis and neurofibromatosis type 1 with a PC or RCC, respectively, these do not cause diagnostic difficulties because of the syndromic features in such cases and have not been reported to cause RAPTAS (23, 24).

### Table 3. Clinical and Molecular Genetic Features of Non-VHL RAPTAS Cases Identified in the Literature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Group A/B</th>
<th>PC/PGL/HNPGL Location (Age)</th>
<th>RCC Tumor Type (Age in Years)</th>
<th>Sex</th>
<th>Tumor of Relative (Age in Years)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHB</td>
<td>c.3G&gt;A (p.Met1Ille)</td>
<td>A+B</td>
<td>PGL (25)</td>
<td>Bilateral RCC (25)</td>
<td>M</td>
<td>RCC, brother (23)</td>
<td>13</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.3G&gt;A (p.Met1Ille)</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (23)</td>
<td>M</td>
<td>RCC, PGL, brother (25)</td>
<td>13</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 3 deletion</td>
<td>A</td>
<td>HNPGL (30)</td>
<td>Unilateral RCC (36)</td>
<td>M</td>
<td>13, 33</td>
<td></td>
</tr>
<tr>
<td>SDHB</td>
<td>c.166-170 del CCTCA</td>
<td>A</td>
<td>PGL (28)</td>
<td>Unilateral RCC (28)</td>
<td>M</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>SDHB</td>
<td>c.423+1G</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC</td>
<td>M</td>
<td>PC, brother (44)</td>
<td>7, 33</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 1 deletion</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCCa (36)</td>
<td>M</td>
<td>RCC, brother (25)a</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>Exon 1 deletion</td>
<td>A+B</td>
<td>PC</td>
<td>Unilateral RCC (42)</td>
<td>F</td>
<td>PGL, sister</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>268C&gt;T (p.Arg90X)</td>
<td>A+B</td>
<td>PGL</td>
<td>Unilateral RCC (61)</td>
<td>M</td>
<td>PGL, son</td>
<td>33</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.286G&gt;A (p.Gly96Ser)</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (52)a</td>
<td>F</td>
<td>RCC, daughter</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.541-2A&gt;G</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (19)</td>
<td>M</td>
<td>PGL, mother</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.689G&gt;A (p.Arg230His)</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (52)</td>
<td>F</td>
<td>PGL, daughter</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.541-2A&gt;G</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (50)</td>
<td>M</td>
<td>RCC, brothera</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>Del exon 1</td>
<td>A</td>
<td>PGL (17)</td>
<td>Unilateral renal oncocytoma</td>
<td></td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.170A&gt;G (p.His57Arg)</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDHB</td>
<td>c.847-50delCTTC</td>
<td>A+B</td>
<td>Unilateral RCC (26)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDHB</td>
<td>c.397C&gt;T (p.Arg133X)</td>
<td>B</td>
<td>No</td>
<td>Unilateral RCC (53)a</td>
<td>F</td>
<td>RCC, son (40)</td>
<td>39</td>
</tr>
<tr>
<td>SDHB</td>
<td>c.3G&gt;A (p.Met1Ille)</td>
<td>B</td>
<td>HNPGL (46)</td>
<td>Bilateral RCC (48,60)</td>
<td>M</td>
<td>Bilateral RCC, mother (48,60)</td>
<td>40</td>
</tr>
<tr>
<td>SDHD</td>
<td>c.239G&gt;T (p.Leu80Arg)</td>
<td>A+B</td>
<td>Bilateral HNPGL (17), PGL(28)</td>
<td>Unilateral RCC (45)a</td>
<td>M</td>
<td>HNPGL, father, PC brother</td>
<td>39</td>
</tr>
<tr>
<td>SDHD</td>
<td>c.308delG (p.Gly103Alafs)</td>
<td>Deletion exon 1+2</td>
<td>A</td>
<td>PC (47)</td>
<td>Unilateral RCC (47)</td>
<td>F</td>
<td>Bilateral PC (28)</td>
</tr>
<tr>
<td>SDHD</td>
<td>Deletion exon 1+2</td>
<td>A+B</td>
<td>Bilateral PC (45)</td>
<td>Unilateral oncocytoma (45)</td>
<td>M</td>
<td>Bilateral PC, brother (28)</td>
<td>8</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male.

aMetastatic disease.

### Discussion

A large case series and literature review demonstrated that non-VHL RAPTAS is genetically heterogeneous. RAPTAS may be caused by germline mutations in six genes (VHL, SDHB, SDHC, SDHD, TMEM127, and MAX) and two further genes, FH and SDHA, have each been reported to predispose to both groups of tumors (PC/PGL/HNPGL and renal tumors) and may yet be described as a cause of RAPTAS. Also, germline mutations in MET cause familial type 1 papillary RCC and recently MET variants have been linked to PC/PGL susceptibility (5).

In both the literature review and case series, SDHB mutations were the most common identified cause of non-VHL RAPTAS. Less frequently, RAPTAS was associated with mutations in other SDHx genes and mutations in TMEM127 and MAX. A limitation of this case series was that all cases had not been tested for mutations in the rarer RAPTAS genes (SDHC, TMEM127, and MAX) and a limitation of the literature review is probable bias against reports of RAPTAS without an identified genetic diagnosis. Nevertheless, we found that there is a substantial group of RAPTAS patients without an identified germline mutation,
suggesting that further RAPTAS genes are still to be identified.

Recently Kopershoek et al. (8) described a germline MAX mutation (a large, complex genomic alteration encompassing the intragenic and promoter regions of MAX and FUT8) in a patient with renal oncocytoma, bilateral PC, and erythrocytosis and two siblings with bilateral PC. In this study, we report the association of RCC with a germline MAX mutation (c.97C>T p. Arg33*). We detected evidence of preferential loss of the wild-type allele in both tumors (PC and RCC) similar to previously reported cases of MAX-related tumors PC/ PGL (25). This finding expands the phenotype associated with MAX mutations and raises the intriguing possibility that MAX may be a candidate gene for inherited RCC [SDHB mutations were originally described in association with PC/PGL/HNPGL (26), then with RAPTAS (20), and then familial RCC-only (16) phenotypes]. Although mutations in all RAPTAS genes are inherited in autosomal dominant manner, mutations in MAX and SDHD show a parent-of-origin–dependent tumorigenesis, and tumors occur almost exclusively following paternal transmission of the mutation. Hence the clinical management and genetic counseling of RAPTAS kindreds with SDHD and MAX mutations will differ from those with mutations in other RAPTAS genes.

We describe the second reported case of a patient with RAPTAS resulting from a mutation in TMEM127. The first report was in a 47-year-old woman (6) with multifocal unilateral PC and a unilateral (clear cell) RCC (6). A germline deletion mutation in TMEM127 (c.308delG) and an additional germline variant in SDHB (159_*184delins25) was identified in this patient, but SDHB immunohistochemistry showed preservation of SDHB expression in both tumors. Histology of the RCC in RAPTAS patient 11 with a TMEM127 mutation demonstrated a clear-cell RCC. Although this is the most common type of RCC, the four additional reported cases of TMEM127-associated RCC were all clear-cell variant RCC (27).

Role of clinical features in suggesting specific genes

In genetically heterogeneous conditions, it is helpful if specific clinical features can guide genetic testing. Clear-cell RCC, PC (less often PGL and rarely HNPGL), and retinal and central nervous system hemangioblastomas (15) (or the presence of pancreatic or renal cysts) should prompt genetic testing for VHL mutations. The occurrence of HNPGL, abdominal PGL, and malignant PPGL or the co-occurrence of wild-type gastrointestinal stromal tumors suggests a possible SDHx mutation. Adrenal PC is more common in VHL disease, whereas (extra-adrenal) PGL with SDHB disease but with a secretory pattern (predominantly noradrenergic) is similar to VHL and SDHX and there are similar features on positron emission tomography computed tomography with tracers such as 18-fluorodeoxyglucose (28).

Indicators of an inherited cancer predisposition syndrome include the occurrence of uncommon/rare tumors in the same individual, related tumor types in close relatives, early age at diagnosis, and the presence of multicentric disease. In patients with RCC, genetic investigation should be considered in sporadic cases age ≤45 years (29). Although the literature review identified patients with non-VHL RAPTAS and a germline mutation had relatively young-onset PC/PGL/HNPGL (mean, 31.8 years; RCC, 41.4 years) in the case series, there was no clear relationship between age at tumor diagnosis and presence/absence of a mutation. Although the difference in MTS (11) between mutation-positive and mutation-negative cases did not reach statistical significance, further studies are required to determine MTS utility in group A RAPTAS cases. Although RAPTAS might in some cases arise coincidentally, we note in two SDHB mutation-positive cases in our series (probands 9 and 18), age at tumor diagnosis was 60 years or older. Therefore, we would suggest that either all cases of RAPTAS should undergo molecular investigation or the cutoff for age at tumor diagnosis for not pursuing genetic testing should not be <70 years.

Role of histology in suggesting specific genes in RAPTAS

Histopathological features may be used to prioritize likely genetic causes of RAPTAS (15). For example, VHL mutations are almost invariably associated with clear-cell RCC (30), and a unique morphology consisting of solid architecture, distinctive intracytoplasmic inclusions, and intratumor mast cells is characteristic of SDHB-deficient RCC (31, 32) (Fig. 2a). Immunohistochemistry is a useful diagnostic adjunct because SDHB-deficient RCC shows negative immunoreactivity (33) (Fig. 2b). Interestingly, proband 2 had evidence of succinate dehydrogenase deficiency on SDHB immunostaining of the PC (Fig. 2c), but immunostaining showed preserved SDHB expression in the RCC tumor. Sequencing of both the PC and RCC tumors in case 2 revealed a somatic mutation in VHL (c.245G>T p.Arg82Leu) in the PC but not the RCC, with no evidence of mutation in SDHA/SDHB/SDHC/SDHD genes. False-positive results using SDHB immunohistochemistry (as apparently occurred in this case) have been reported for patients with germline VHL mutations (34) (Fig. 2d). A potential alternative explanation for the discrepant SDHB immunohistochemistry results in case 2 is that the first hit is an undetected germline VHL mutation (e.g., intronic mutation, copy number alteration) and that
the somatic \textit{VHL} missense mutation in the PCC was the “second hit.” However, the RCC histology was a papillary (Fig. 2c), whereas renal tumors in VHL disease are clear cell (18, 35). Nevertheless, it is important to consider that VHL mutations can lead to false-positive results on SDHB immunohistochemistry (34); therefore, we recommend that those patients with RAPTAS, without a detectable germline mutation in \textit{SDHx}, but with loss of SDHB immunoreexpression on tumor studies, undergo genetic screening for \textit{VHL} mutations (Fig. 3).

\textbf{Molecular pathways implicated in different genetic causes of RAPTAS}

Transcriptome profiling separates inherited PC/PGL into two categories (5). First, the “pseudohypoxic cluster” with the upregulation of hypoxia signaling pathways and “cluster 2” is characterized by an upregulation of kinase signaling pathways (5). \textit{VHL}, \textit{FH}, or \textit{SDHx}-associated PC/PGL fall into the pseudohypoxic cluster 1 (5). SDHx and FH inactivation leads to the accumulation of oncometabolites such as succinate and fumarate that inhibit alpha ketoglutarate dependant dioxygenase enzymes, promoting stabilization of hypoxia inducible factor complex (5) and inhibiting histone and DNA demethylation enzymes, resulting in DNA hypermethylation (36).

Cluster two gene mutations (\textit{RET}, \textit{NF1}, \textit{TMEM127}, \textit{MAX}) activate the MAPK and phosphatidylinositol 3-kinase–AKT–mTOR pathways (5). \textit{MAX} is a protein that contains a basic helix loop helix zipper commonly involved in a complex formation and sequences in the promoter region of hundreds of genes encoding for proteins essential in cellular metabolism and angiogenesis (37).

\textbf{Investigation of potential RAPTAS patients}

In study group A patients, one-third presented with PC/PGL/HNPGL and were subsequently diagnosed with an RCC. The longest interval between the presenting tumor (abdominal PGL) and diagnosis of RCC was 16 years (proband 8). For the other three patients, mean interval between the first tumor and RCC was 4 years (median, 2 years; range, 1 to 9 years). Recently published European guidelines recommend a 10-year follow-up for patients with sporadic PC and life-long follow-up for patients with PGL/HNPGL or those patients with a confirmed genetic predisposition. The recommendations for follow-up include biochemical and radiological surveillance that would include abdominal imaging capable of detecting renal tumors. Data from this study suggest that this surveillance protocol will facilitate the detection of patients with RAPTAS (38).

Patients meeting our clinical criteria for RAPTAS should be referred for genetic testing. If gene panel testing is not available/undertaken, then single-gene testing should be prioritized as suggested in Fig. 3. It is important

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{flowchart.png}
\caption{Flowchart of the recommended genetic evaluation of potential RAPTAS kindreds. MLPA, multiple ligation-dependant probe amplification.}
\end{figure}
that SDHB mutation analysis includes investigation for exonic deletions/duplications because the literature review revealed a higher than expected proportion of deletions in SDHB-associated RAPTAS. Mutation-positive cases should receive appropriate follow-up and surveillance. The clinical benefits of identifying germline genetic variants in patients with non-VHL RAPTAS also include family screening, and, in the future, genetic classification may facilitate a personalized treatment approach.

**Conclusion**

We described the largest cohort to date of non-VHL RAPTAS and have undertaken a literature review of reported cases. The term RAPTAS (rather than inherited PC-PC) emphasizes that many cases may be sporadic with no family history and that not all cases may have a genetic origin. We provide guidelines for genetic testing in suspected RAPTAS and for clinical diagnostic criteria that include both sympathetic and parasympathetic PGL (with PC) in the criteria and both malignant (RCC) and benign (oncocytoma) renal tumors. Application of whole-exome and whole-genome sequencing to undiagnosed RAPTAS cases will provide further insights into the molecular mechanisms of this association and improve the management of these cases.

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**Correspondence and Reprint Requests:** Ruth T. Casey, MD, Department of Academic Medical Genetics, Box 238, Level 6 Addenbrooke’s Hospital, NHS Foundation Trust, Hills Road, Cambridge CB2 OQQ, United Kingdom. E-mail: ruthcasey232@gmail.com.

**Disclosure Summary:** The authors have nothing to disclose.

**References**


A case of a metastatic SDHA mutated paraganglioma re-presenting twenty-three years after initial surgery

Dear Editor,

We have read with great interest the recent article by Tufton and coworkers reporting on the risk of metastasis in patients with paraganglioma (PGL) tumours associated with germline SDHA mutations (Tufton et al. 2017). Herein, we report a further case of a malignant PGL in a 46-year-old man with a succinate dehydrogenase complex flavoprotein subunit A (SDHA) germline mutation (c.91C>T, p.Arg31*). In the case we describe, following the initial surgical removal of a left-sided retroperitoneal PGL, twenty-three years elapsed before the development of a bony metastasis in the eighth left rib. This observation is similar to that of Tufton and coworkers who reported two patients, who developed metastatic disease in 16 and 37 years, respectively, following initial diagnosis (Tufton et al. 2017). During investigations for this case, we found that the rib metastasis was avid on 18-fluorodeoxyglucose (FDG) positron emission tomography (PET) computed tomography (CT), but not avid on radiolabeled 1131 metaiodobenzylguanidine (MIBG) imaging (Fig. 1). This observation of SDHA-related malignant PGL is in keeping with SDHB- and SDHD-associated PGL, in which reduced avidity on MIBG imaging is due to reduced tumoural expression of noradrenaline transporters (Timmers et al. 2007). This clinical report supports the recent literature that suggests a risk of malignancy in patients with SDHA mutated PGL (Bausch et al. 2017, Casey et al. 2017, Tufton et al. 2017). The prolonged time interval between diagnosis and development of metastases in our case suggests that SDHA-related tumours are slow growing but it does support long-term surveillance programmes for patients with germline SDHA mutations.

Although mutations in SDHB, SDHC and SDHD gene subunits were first identified as predisposition genes for the development of phaeochromocytoma (PC) and PGL tumours over 15 years ago, SDHA was first associated with PGL only seven years ago (Burnichon et al. 2010) and, more recently, with wild-type gastrointestinal stromal tumours (WT GIST) (Evenepoel et al. 2015). It is now recognised that the SDHA gene is the most common SDHx germline mutation implicated in development of SDH-deficient WT GIST (Boikos et al. 2016). Recently, the European-American-Asian Pheochromocytoma-Paraganglioma Registry Study Group reported on 34 index cases with germline SDHA mutations and PC or PGL tumours. This group described a high prevalence of head and neck PGL in the SDHA cohort 15/34 (44%) and metastatic disease was reported in 4/34 (12%) (Bausch et al. 2017).

Here, we report the case of a 46-year-old man who first presented at age 23 years with headache, heat intolerance and abdominal pain. He was subsequently diagnosed with a left-sided retroperitoneal paragangliomama, which was surgically resected. The man had no family history of endocrine tumours and no additional relevant medical history. Following surgery, the patient was surveyed in primary care with annual urinary metanephrine testing. Twenty-three years later he was referred to our specialist neuroendocrine tumour service at Cambridge University Hospital NHS Foundation Trust, due to an elevated urinary normetanephrine level (urinary normetanephrine 5870 nmol/24 h, reference range 0–4900; urinary metanephrine 756 nmol/24 h, reference range 0–2000), which was first observed 3 months earlier. On review, the patient denied symptoms suggestive of catecholamine excess and, importantly, denied any of the symptoms that he reported at the time of his initial presentation. Review of systems revealed that his only complaint was left side rib pain. Previous investigations in primary care included a plain chest radiograph which did not reveal any abnormality.

We performed plasma metanephrine testing and found an elevated normetanephrine level (2864 µmol/L, reference range <1000 µmol/L) in the context of normal plasma metanephrine (197 µmol/L, reference range <600 µmol/L) and methoxytyramine measurements (95.6 µmol/L, reference range <180 µmol/L). In light of the elevated normetanephrine level in urine and plasma, CT of the
neck, thorax, abdomen and pelvis was performed and a metastatic deposit in the eighth left rib with no evidence of additional disease was identified (Fig. 1A). An $^{131}$I-MIBG scan was performed both for staging purposes and to evaluate suitability for $^{131}$I-MIBG therapy. Interestingly, the metastatic deposit was not avid (Fig. 1C). An $^{18}$F-FDG PET CT confirmed avidity (maximum standard uptake value of 19.1) in the left rib lesion (Fig. 1B) but no additional disease was identified. The patient was referred for a thoracotomy and surgical resection of the 8th rib after appropriate pre-operative optimisation with alpha-blockade. Subsequently, histological analysis confirmed the diagnosis of a metastatic paraganglioma with SDHB immunonegativity. SDHA immunohistochemistry was not performed (Fig. 1D).

Next generation sequencing of SDHA, SDHB, SDHC, SDHD, SDHAF2, MAX, TMEM127, VHL, RET and FH was performed on germline DNA from this patient after obtaining informed consent. The Illumina Trusight-One assay was used for sequencing and a mean coverage depth of $>20$-fold was achieved for 98% of the regions sequenced. Whole exon deletions, duplications and large rearrangements are not detected using this method and multiple ligation probe analysis was performed for VHL, SDHB, SDHC and SDHD. This testing identified a pathogenic truncating mutation in the SDHA gene (c.91C>T, p.Arg31*), which was confirmed by Sanger sequencing.

Post-operatively, repeat plasma metanephrine levels returned to normal (normetanephrine, 391 pmol/L; metanephrine, 180 pmol/L). This patient will remain under regular clinical follow-up for the development of synchronous or metachronous tumours. Given his germline SDHA mutation status and previous disease avidity on $^{18}$F-FDG PET CT, going forward, this will be the preferred surveillance imaging modality if there is no detectable disease on conventional cross-sectional imaging using CT/ MRI, in the presence of elevated plasma metanephrine or methoxytyramine levels.

In summary, the case we describe provides additional evidence for the risk of metastatic disease in SDHA mutated PGL. Moreover, our report highlights the utility of $^{18}$F-FDG PET CT in the detection of metastatic disease in patients with SDHA mutations, as has previously been demonstrated in cases of SDHB and SDHD related tumours (Timmers et al. 2007). Interestingly, and with relevance to optimal surveillance strategies for patients with germline SDHA mutations, the significant lag period (23 years) between initial presentation and the development of metastatic disease described in our case was also observed in two other cases of SDHA mutated malignant PGL (Tufton et al. 2017). The incidence of metastatic disease in SDHB related PC and PGL has been reported in two studies as 19% (Benn et al. 2006) and 16% (Srirangalingam et al. 2008) over a mean
follow-up of 48 and 70 months, respectively. These data suggest that the development of metastatic disease may occur earlier in the disease course of SDHB-associated PGL/PC. Further study is required to define the risk of malignant disease in SDHA-related PGL/PC and the median interval for the development of malignancy. Recent literature suggests that life-long surveillance, as recently recommended by the European Society of Endocrinology (Plouin et al. 2016), is crucial for patients with SDHA gene mutations, in addition to those patients with mutations in other SDH subunits (SDHB/SDHC/SDHD). However, we acknowledge that prospective studies with extended follow-up periods of 15–20 years may be necessary to reveal the true incidence of metastasis in SDHx related PGL and to stratify individual surveillance protocols for patients based on the SDHx germline subunit mutation.

Ruth T Casey1,2
Benjamin G Challis2
Alison Marker3
Deborah Pittfield2
Heok K Cheow4
Ashley Shaw4
Soo-Mi Park3
Helen L Simpson5
Eamonn R Maher1

1Department of Medical Genetics, University of Cambridge and NIHR Cambridge Biomedical Research Centre and Cancer Research UK Cambridge Centre, Cambridge, UK
2Department of Endocrinology, Cambridge University NHS Foundation Trust, Cambridge, UK
3Department of Histopathology, Cambridge University NHS Foundation Trust, Cambridge, UK
4Department of Radiology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
5Department of Diabetes and Endocrinology, University College London Hospitals, NHS Foundation Trust, London, UK

(Correspondence should be addressed to R Casey; email: rc674@medschl.cam.ac.uk)

Declaration of interest
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References


Translating In Vivo Metabolomic Analysis of Succinate Dehydrogenase–Deficient Tumors Into Clinical Utility

Purpose Mutations in the mitochondrial enzyme succinate dehydrogenase (SDH) subunit genes are associated with a wide spectrum of tumors, including pheochromocytomas and paragangliomas, GI stromal tumors, renal cell carcinomas, and pituitary adenomas. SDH-related tumorigenesis is believed to be secondary to accumulation of the oncometabolite succinate. Our aim was to investigate the potential clinical applications of proton-1 magnetic resonance spectroscopy (1H-MRS) in a range of suspected SDH-related tumors.

Patients and Methods Fifteen patients were recruited to this study. Respiratory-gated single-voxel 1H-MRS was performed at 3T to quantify the content of succinate at 2.4 ppm and choline at 3.22 ppm.

Results A succinate peak was seen in six patients, all of whom had germ line SDHx mutations or loss of SDHB by immunohistochemistry. Succinate peaks were also detected in two patients with metastatic wild-type GI stromal tumors and no detectable germ line SDHx mutations but with somatic epimutations in SDHC. Three patients without tumor succinate peaks retained SDHB expression, consistent with SDH functionality. In six patients with borderline or absent peaks, technical difficulties such as motion artifact rendered 1H-MRS difficult to interpret. Sequential imaging in a patient with a metastatic abdominal paraganglioma demonstrated loss of the succinate peak after four cycles of [177Lu]DOTATATE, with a corresponding biochemical response in normetanephrine.

Conclusion This study has demonstrated the translation into clinical practice of in vivo metabolomic analysis using 1H-MRS in patients with SDH-deficient tumors. Potential applications include noninvasive diagnosis and disease stratification, as well as monitoring of tumor response to targeted treatments.

INTRODUCTION

The succinate dehydrogenase (SDH) enzyme is composed of four subunits (A to D) and plays a key role in the Krebs cycle and oxidative phosphorylation.1 In the past two decades, germ line mutations in the genes encoding the four SDH subunits (SDHA, SDHB, SDHC, and SDHD), collectively known as SDHx, have emerged as an important cause of human neoplasia and a paradigm for the role of disordered cellular metabolism in oncogenesis.2-7 SDHx mutations were described initially in association with head and neck paragangliomas (PGLs; derived from sympathetic ganglia) and in pheochromocytomas and PGLs (PPGLs; derived from sympathetic ganglia and often secreting catecholamines).2,3 It is now recognized that approximately 40% of patients with PPGLs harbor germ line mutations in inherited PPGL genes, and SDHx mutations comprise the most common cause of PPGL predisposition.8 In addition, germ line SDHB mutations are associated with a high risk of malignancy in PPGLs.9 Other tumor types associated with SDHx mutations include GI stromal tumors (GISTs) and renal cell carcinomas (RCCs).10-12 GISTs are mesenchymal tumors of the GI tract and in adults are usually associated with somatic activating mutations in
the KIT or PDGFRA gene. However, GISTs without KIT or PDGFRA gene mutations, known as wild-type GISTs (wtGISTs), account for 15% of adult and 85% of pediatric GISTs, and recent studies suggest that up to 88% of wtGISTs are SDH deficient. A wtGIST with SDH deficiency may harbor a germ line SDHx mutation (75% of cases) or an SDHC gene epimutation with hypermethylation of the promoter region. Only approximately one third of patients with SDH-deficient wtGISTs achieve disease stabilization with imatinib therapy, and the risk of metastatic disease is higher with SDH-deficient GISTs compared with conventional GISTs. SDHx-associated RCC may present in patients with a personal or family history of PPGL or may present with an RCC-only phenotype. Finally, germ line SDHx mutations have been described in rare patients with pituitary adenomas. Despite recent advances in the understanding of the SDHx genes, there are many areas of unmet clinical need, including a lack of robust biomarkers to predict aggressive biologic behavior and inform clinical surveillance and management.

Succinate has been shown to be elevated by 100-fold in SDHx-mutated PPGLs ex vivo compared with non–SDHx-mutated PPGLs. Recently, in vivo detection of succinate by magnetic resonance spectroscopy (MRS) was reported in two patient cohorts with SDH-deficient PPGLs. Similarly, the noninvasive detection of 2-hydroxyglutarate with proton-1 MRS (1H-MRS) has been demonstrated within gliomas in patients with gain-of-function mutations in another citric acid cycle enzyme, isocitrate dehydrogenase. The ability to measure succinate in vivo has a number of important potential clinical applications, including early identification of SDH deficiency, which could enable tailored patient surveillance and management. In vivo detection of succinate accumulation could also serve to verify genetic variant pathogenicity in the era of next-generation sequencing. The aim of this study was to investigate the role of 1H-MRS in detecting abnormally elevated succinate in vivo in patients with suspected SDH-deficient tumors, expanding the applications of 1H-MRS in SDH-deficient tumorigenesis to include GIST and pituitary adenoma for the first time and to explore the technique as a potential noninvasive biomarker of treatment response.

PATIENTS AND METHODS

Patient Selection

This study was performed as a prospective case series, and participants were recruited from a dedicated neuroendocrine tumor clinic and a national pediatric and adult wtGIST clinic of the Cambridge University National Health Service Foundation Trust. Suitable patients were identified based on SDHx germ line status, suspicious clinical phenotype (metastatic PPGL, PGL, or wtGIST), and/or immunohistochemistry of tumor tissue showing absent SDHB immunostaining. A minimum tumor size threshold of 1.5 cm was applied for inclusion in the study. All participants provided written informed consent, and the study was approved by the Cambridge South Research Ethics Committee.

MRS Analysis

Both SAGE (GE Healthcare, Waukesha, WI) and LCModel (s-provencher.com) spectroscopy analysis programs were used to reconstruct, analyze, and display spectra. For each metabolite, LCModel reports both peak area and estimated uncertainty in fitting of the peak (percent standard deviation [SD]). This uncertainty measure was used to stratify the results using the following algorithm: 1) if percent SD of choline was > 15%, the spectrum was discarded as a technical failure, because it was assumed that choline should be detectable in a metabolically active tumor, such that SD > 15% would indicate probable data quality issues; 2) succinate detection was taken as positive if its percent SD was < 50% and negative if it was > 50%. The succinate-to-choline ratio (SCR) was quantified, the full width at half maximum height of the water peak in Hz was measured in SAGE and recorded as an additional data quality metric, and an expert spectroscopist was asked to rate whether detected succinate peaks were convincing or unconvincing based on data displayed both in LCModel and in SAGE.

Information on statistical methods, 1H-MRS data acquisition, germ line genetic analysis, SDHB immunohistochemistry, SDHC hypermethylation analysis, and measurement of succinate in ex vivo tissue samples is provided in the Data Supplement.
RESULTS

Patients and Clinical Phenotypes

Fifteen patients (six women and nine men; mean age, 40 years [range, 21 to 80 years]) were studied. Seven wtGISTs, three unilateral adrenal pheochromocytomas, three abdominal PGLs, a large left glomus PGL, and a nonfunctioning pituitary macroadenoma were examined. Nine patients (60%) had metastatic disease: six with wtGISTs, two with abdominal PGLs, and one with a unilateral pheochromocytoma. The liver was the most common site for metastases (seven [77.7%] of nine patients). Three patients had multicentric primary tumors, including patient 5, who presented with a metastatic wtGIST and was subsequently diagnosed with a 1.9-cm carotid body PGL (Fig 1D); patient 9, with an abdominal PGL and a small left-sided 1.5-cm carotid PGL (Fig 2B); and patient 8, with a large left-sided glomus PGL and a 2-cm prolactin-secreting pituitary adenoma (Data Supplement).

Only two patients had positive family histories (patients 2 and 6; Table 1).

Genotype

A germ line mutation in an SDHx gene was identified in nine (60%) of 15 patients: five in SDHB (four missense variants and one truncating variant) and four in SDHA (one missense and three truncating). Two additional patients were found to have somatic SDHC epimutations (Table 1).

1H-MRS Succinate Analysis

The 1H-MRS characteristics of the 15 patients are listed in the Data Supplement. Mean size of the tumors selected for spectra acquisition was 5.5 cm (median, 3.3 cm; range, 1.8 to 12 cm). The liver was the most common site to be assessed (n = 6), but good-quality spectra were also obtained from a pituitary tumor (n = 1) and PPGLs (n = 5). Patients were divided into four...
groups according to whether a succinate tumor peak was present, a succinate tumor peak was absent, a borderline peak was detected, or technical failure prevented interpretation of the spectra.

**Succinate peak detected.** Succinate was detected at 2.4 ppm in six patients (40%). The mean SCR in these patients was 1.3 (SD ± 0.71), and the mean tumor size in these six patients with reliable succinate peak detection was 4.8 cm (SD ± 2.94 cm; range, 2.3 to 9 cm). The in vivo detection of succinate on 1H-MRS correlated with tumor SDH deficiency; four of the six patients had germline SDHx mutations (Fig 2), and loss of SDHB expression on immunohistochemistry and a somatic SDHC epimutation were detected in two of the six patients (Fig 3).

**Borderline succinate peak detected.** A borderline succinate peak was detected in two patients. Patient 8, with a germ line SDHB mutation (c.600G>T p.Trp200Cys) and a glomus PGL, demonstrated an SCR of 1.19; however, the line width (29 Hz) was so broad as a result of the proximity of metallic dental work that the peak assignments were not reliable (Data Supplement). Patient 7, with a metastatic pheochromocytoma and no detectable germ line SDHx mutation, demonstrated an SCR of 0.18, but LCM detected a small succinate peak at 2.4 ppm; this patient did not undergo surgery or diagnostic biopsy, and therefore, no tissue was available for further analysis; we classified this case as borderline.

**No succinate peak.** No succinate peak was detected in three patients. Patient 4 had a metastatic wtGIST with no detectable germ line SDHx mutation and preserved SDHB protein expression in the tumor tissue; choline was confidently fitted on LCM, but no succinate was seen. Patient 6 demonstrated a good-quality spectrum from the remnant pituitary adenoma; choline was detected on LCM and SAGE processing, but no succinate was detected, and this finding was consistent with the preservation of SDHB protein expression in the pituitary tumor by immunohistochemistry (Fig 4).

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Fig 2. (A) T2-weighted magnetic resonance (MR) image showing a large nonsecretory abdominal paraganglioma (PGL) from patient 9 (arrow). (B) Axial fused [18F]fluorodeoxyglucose positron emission tomography (PET)/computed tomography image. (C) Proton-1 MR spectra demonstrating a succinate peak at 2.4 ppm. The corresponding coronal maximum-intensity projection PET image demonstrates a synchronous left-sided carotid PGL. (D) Spectra acquired by high-resolution magic angle spinning in vitro on the PGL tumor sample, again confirming a succinate peak at 2.4 ppm.
Patient 10 had no detectable germ line SDHx mutation and preserved SDHB protein expression in the tumor tissue; choline was detected in the tumor on 1H-MRS, but succinate was not detected.

Technical failure. Technical failure occurred in four patients (26%). Patient 12 demonstrated no reliable detection of succinate or choline because of motion artifact and a low signal-to-noise ratio (SNR), which probably resulted from inconsistent breathing, because the voxel was at the edge of the liver. A small rib metastasis was imaged in patient 13, but only a pure lipid spectrum was obtained from this challenging location. A metastasis on the edge of the liver was imaged in patient 14, where again inconsistent respiration probably led to displacement of the voxel into adjacent adipose tissue. Finally, patient 15 had a unilateral pheochromocytoma with a large volume of blood, the paramagnetic properties of which may have affected acquisition, leading to a low SNR (Data Supplement).

Table 1. Clinical Characteristics of the Cohort

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genetic Mutation</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Primary Tumor</th>
<th>Metastatic Disease</th>
<th>Site of Metastasis</th>
<th>Family History</th>
<th>Other Primary Tumor</th>
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<tbody>
<tr>
<td>1</td>
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<td>GIST</td>
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<td>Liver, lung</td>
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<tr>
<td>2</td>
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<td>53</td>
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<td>Lymph nodes, bone</td>
<td>Yes, mother (GIST)</td>
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<tr>
<td>c.268C&gt;T p.(Arg90*)</td>
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<td></td>
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<tr>
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<td>GIST</td>
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<td>PA</td>
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<td>M</td>
<td>70</td>
<td>PC</td>
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<td>Liver, bone</td>
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Abbreviations: GIST, GI stromal tumor; NA, not applicable; PA, pituitary adenoma; PC, pheochromocytoma; PGL, paraganglioma.
Sequential 1H-MRS Succinate Analysis

Patient 2 with a metastatic PGL to the lung, bone, and lymph node and a germ line SDHB mutation (c.268C>T p.Arg90*) underwent 1H-MRS on a large pelvic nodal metastasis before treatment with four cycles of lutetium-177–labeled peptide receptor radionuclide therapy. Succinate and choline peaks were detected, with an SCR of 1.32 (Figs 5A and 5B). After four cycles of treatment, a repeat 1H-MRS examination on the same pelvic nodal metastases revealed a choline peak but no succinate peak (Fig 5C). Although the magnetic resonance imaging features of the metastatic lesions were unchanged pre- and post-treatment, the loss of a succinate peak was correlated with a reduction in plasma normetanephrine levels (from 1,861 to 1,193 pmol/L) and tumor avidity on [18F]fluorodeoxyglucose ([18F]FDG) positron emission tomography (PET)/computed tomography (CT; standardized uptake value: pretreatment, 16.1; post-treatment, 9.3; Figs 5D to 5F). The detection of choline on the acquired spectra both before and after treatment indicates that tumor necrosis is unlikely to account for the absent succinate peak post-treatment.

A sequential 1H-MRS study was performed for patient 5 because of evidence of progressive disease on surveillance CT, despite treatment with a multikinase inhibitor, regorafenib. Serial 1H-MRS demonstrated a larger succinate peak compared with the first study (Fig 1), and this correlated with the [18F]FDG avidity on PET/CT pretreatment and 10 months post-treatment, which demonstrated an increase in disease burden and avidity (standardized uptake value, 15.1 and 27.1, respectively; Figs 1F and 1G).

Repeatability of 1H-MRS was evaluated in two patients by investigating different tumor deposits during the same study examination (patient 5) and the same tumor deposit twice during the same study examination (patient 1). The results for SCR were almost identical in these two patients, suggesting good test reproducibility (Data Supplement).

DISCUSSION

This proof-of-principle study demonstrates that detection of a succinate peak and an increased SCR was specific for a variety of SDH-deficient tumor types. All six tumors with a positive succinate peak and elevated SCR were associated with a germ line SDHx mutation (n = 4) or an SDHC epimutation (n = 2). In addition, the three patients with absent succinate peaks but
adequate 'H-MRS demonstrated preservation of SDHB expression in the tumors analyzed. Our findings are complementary to a previous study in which 'H-MRS was applied in nine patients with PGLs, and succinate peaks were detected in all five with SDHx mutations but not in the four patients without mutations.16 We demonstrate for the first time to our knowledge that 'H-MRS can also be used to determine the SDH status of GISTs and pituitary adenomas and that succinate peaks can be detected in SDH-deficient tumors with epigenetic inactivation of SDHC. 'H-MRS might have clinical utility in a wide variety of situations. Potential diagnostic applications of this new approach include: assessing the pathogenicity of patients with germ line SDHx variants of uncertain significance and potentially SDH-related tumors; investigating possible metastatic lesions (eg, in the liver) in patients with germ line SDHx mutations and primary SDH-deficient tumors; assessing patients with multiple primary tumors to determine if all are SDH deficient; identifying patients without detectable germ line SDHx mutations who might benefit from specialist genetic investigations, such as SDHC promoter methylation status; and assessing SDH tumor status preoperatively, particularly for patients with possible wtGISTs, because standard adjuvant treatment with imatinib has proven to be less effective in patients with SDH-deficient disease.12

Notably, here we use the presence of a choline signal as an internal control for viable tissue to discriminate technical failures from a negative finding. To avoid issues of partial volume effects within smaller tumors, the voxel for MRS analysis was chosen to fully include tumor where possible. We did not detect a statistically significant correlation between tumor size and SCR, although there was a trend toward significance. This trend is the opposite of what would be expected if necrosis were artificially lowering the overall succinate levels in large tumors and therefore suggests that the method is measuring real differences in succinate, which are independent of tumor size. However, we recommend using a size threshold of > 2 cm where possible to improve the sensitivity of the test.

Fig 4. (A) Coronal T1-weighted magnetic resonance (MR) image demonstrating a remnant pituitary adenoma in patient 6 (white arrow). (B) Spectra acquired from the pituitary tumor with proton-1 MR spectroscopy, with evidence of choline detection but no succinate. (C) Succinate dehydrogenase B (SDHB) immunohistochemistry demonstrating preservation of the SDHB protein performed on a section of tumor tissue debulked from the pituitary tumor.
There is increasing interest in understanding the metabolic adaptations that occur during tumorigenesis and how these might be exploited for novel therapeutic interventions. Increased production of lactate during aerobic glycolysis in most cancers, or the Warburg effect, is the best-known example of this. SDH-related cancers provide a paradigm for investigating tumor metabolism, because succinate is thought to act as an oncometabolite and to drive tumorigenesis. Succinate inhibits 2-oxoglutarate-dependent dioxygenases, including DNA and
In the future, 1H-MRS may be complemented to differentiate intracellular from extracellular metabolites. Succinate, and it can be challenging to differentiate enzyme flux in vivo.26

The availability of sensitive noninvasive biomarkers would greatly facilitate precision medicine–based clinical trials. Imaging with [18F]FDG PET to measure the uptake and phosphorylation of a glucose analog to probe the increased glucose use that occurs in many metabolically active cancers is a useful form of in vivo metabolic imaging and has been used for the detection of primary and metastatic diseases in many tumor types, including PPGL and GIST,24,25 and is in widespread clinical use. However, despite being a sensitive imaging tool, [18F]FDG PET lacks specificity and cannot differentiate individual metabolites. 1H-MRS is highly specific and allows in vivo detection of individual metabolites without the use of ionizing radiation; however, 1H-MRS is significantly less sensitive than PET, which could limit the detection of low levels of succinate, and it can be challenging to differentiate intracellular from extracellular metabolites. In the future, 1H-MRS may be complemented by other techniques, such as hyperpolarized carbon-13 MRS, which can increase 13-MRS SNR by several orders of magnitude, allowing assessment of enzyme flux in vivo.25

We show that 1H-MRS could be a valuable tool for the assessment of tumor response in the context of radionuclide and other therapies, because alterations in succinate levels were detected despite stable appearance of the tumor diameters. This important application of 1H-MRS could be expanded to include other tumors with specific metabolic defects, including fumarate hydratase–deficient tumors,27 IDH1-mutant tumors,18 and the recently identified malate dehydrogenase 2–deficient tumors.28 However, important limitations of in vivo metabolomic analysis using 1H-MRS were also revealed by our study; for example, spectral quality was poor in close proximity to metal dental work, in areas adjacent to air spaces such as the lung, and in bone metastases and was susceptible to motion artifact. In this study, the technical failure rate was 26%, which is similar to the failure rate reported in previous studies using 1H-MRS.26 Importantly, no patient cases were excluded from this prospective study, with the intention that this would inform the translation of this imaging modality into clinical practice. On the basis of the evidence from this exploratory study, we recommend that tumors be selected for 1H-MRS analysis based on the following: ideally the largest tumor deposit but at least > 2 cm in size; tumors located close to bone or lung should be avoided; tumors with significant necrosis or hemorrhage should be avoided; superficial tumor deposits should be selected preferentially; and respiratory-triggered acquisition should be used for tumors in the upper abdomen, such as hepatic metastases.

Although the use of 1H-MRS as a diagnostic tool is likely to be limited to specialist centers, the number of scan averages in our study during spectral acquisition was less than half those reported in a previous study26 (200 vs 512), without demonstrating a reduction in sensitivity. Using fewer scan averages reduces the acquisition time, making it more cost effective and convenient for the patient. This is a particularly important consideration if this imaging technique is to be considered for routine clinical practice or for sequential follow-up as part of a clinical trial. Furthermore, this imaging modality could be used to investigate other metabolically driven tumors.

In conclusion, this study is the largest to date to our knowledge to evaluate 1H-MRS in patients with SDH deficiency. It reveals that 1H-MRS has the potential to be used as a noninvasive biomarker in the precision management of SDH-deficient disease and could have a role as a biomarker of successful treatment response. Lessons learned from this study could be applied to other similar metabolically driven tumors.

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AUTHOR CONTRIBUTIONS

Conception and design: Ruth T. Casey, Mary A. McLean, Olivier Giger, Eamonn R. Maher, Ferdia A. Gallagher

Financial support: Ruth T. Casey, Ferdia A. Gallagher and Eamonn R. Maher

Provision of study material or patients: Ruth T. Casey, Benjamin G. Challis, Deborah Pitfield, Helen L. Simpson, Venkata R. Bulusu, Lisa Happerfield, Soo-Mi Park, Alison Marker, Eamonn R. Maher

Collection and assembly of data: Ruth T. Casey, Mary A. McLean, Basetti Madhu, Benjamin G. Challis, Rogier ten Hoopen, Thomas Roberts, Graeme R. Clark, Deborah Pitfield, Helen L. Simpson, Venkata R. Bulusu, Kieran Allison, Soo-Mi Park, Olivier Giger, Ferdia A. Gallagher

Data analysis and interpretation: Ruth T. Casey, Mary A. McLean, Basetti Madhu, Benjamin G. Challis, Lisa Happerfield, Soo-Mi Park, Alison Marker, Olivier Giger, Ferdia A. Gallagher

Manuscript writing: All authors

Final approval of manuscript: All authors

Accountable for all aspects of the work: All authors

AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Ruth T. Casey
No relationship to disclose

Mary A. McLean
Stock and Other Ownership Interests: Veryan Medical
Patents, Royalties, Other Intellectual Property: Patent for stent design for arterial bypass graft

Basetti Madhu
No relationship to disclose

Benjamin G. Challis
No relationship to disclose

Rogier ten Hoopen
No relationship to disclose

Thomas Roberts
No relationship to disclose

Graeme R. Clark
No relationship to disclose

Deborah Pitfield
Travel, Accommodations, Expenses: Novartis, Ipsen

Helen L. Simpson
No relationship to disclose

Venkata R. Bulusu
Honoria: Pierre Fabre
Consulting or Advisory Role: Bayer HealthCare Pharmaceuticals, ARIAD Pharmaceuticals

Speakers’ Bureau: Pfizer
Travel, Accommodations, Expenses: Pierre Fabre, TEVA Pharmaceuticals Europe

Kieran Allison
No relationship to disclose

Lisa Happerfield
No relationship to disclose

Soo-Mi Park
No relationship to disclose

Alison Marker
No relationship to disclose

Olivier Giger
Stock and Other Ownership Interests: Cambridge Pathology

Eamonn R. Maher
Honoria: Alexion Pharmaceuticals
Travel, Accommodations, Expenses: Alexion Pharmaceuticals

Ferdia A. Gallagher
Research Funding: GE Healthcare, GlaxoSmithKline, GlaxoSmithKline (I)
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Affiliations

Ruth T. Casey, Graeme R. Clark, Soo-Mi Park, and Eamonn R. Maher, University of Cambridge; Ruth T. Casey, Benjamin G. Challis, Rogier ten Hoopen, Venkata R. Bulusu, Kieran Allison, Alison Marker, Olivier Giger, Thomas Roberts, Lisa Happerfield, Ferdia A. Gallagher, and Deborah Pitfield, Cambridge University NHS Foundation Trust; Mary A. McLean and Basetti Madhu, Cancer Research UK Cambridge Institute, Cambridge; and Helen L. Simpson, University College London Hospitals, NHS Foundation Trust, London, United Kingdom.
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