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Role of succinate dehydrogenase in pheochromocytomas and paragangliomas

PhD thesis

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APPENDIX

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Abbreviations

ATP	adenosine triphosphate
CAC	Citric acid cycle
COV	Coefficient of variance
CT	Computer Tomography
ETC	Electron transport chain
FAD	Flavine adenine dinucleotide
^{18}F-FDG-PET	2-^{18}F-fluoro-2-deoxy-D-glucose position emission tomography
FH	Fumarate hydratase
FMTC	Familial Medullary Thyroid Cancer
FPGL	Familial paraganglioma syndrome
GABA	Gamma aminobutyric acid
GC-MS	Gas Chromatography-Mass Spectrometry
HIF 1α	Hypoxia Inducible Factor
H_2O_2	Hydrogen peroxide
KIF1Bβ	Kinesin family member 1B
MDH	Malate dehydrogenase
MEN	Multiple Endocrine Neoplasia
MIBG	Metaiodobenzylguanidine scintigraphy
MPC	Mouse pheochromocytoma cells
MRI	Magnetic Resonance Image
MTC	Medullary Thyroid Cancer
MTT	Mouse tumor tissue
NADH	Reduced form of nicotinamide adenine dinucleotide
NF	Neurofibromatosis
PGL	Paraganglioma
PHD	Prolyl hydroxylase
PHEO	pheochromocytoma
RET	Rearranged during transfection

RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
SD	Standard deviation
SDH	Succinate dehydrogenase
SDHA	Succinate dehydrogenase subunit A
SDHB	Succinate dehydrogenase subunit B
SDHC	Succinate dehydrogenase subunit C
SDHD	Succinate dehydrogenase subunit D
SDHx	Succinate dehydrogenase subunits
VHL	von Hippel-Lindau

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1. INTRODUCTION

1.1. Succinate dehydrogenase and function

Succinate dehydrogenase (SDH) described first by Albert Szent-Györgyi in the middle of the 1930's is part of both the citric acid cycle (CAC) and respiratory electron transfer chain (ETC)/oxidative phosphorylation. [1] SDH catalyzes the oxidation of succinate to fumarate in the mitochondrial matrix and transfers electrons to ubiquinone without pumping protons across the mitochondrial inner membrane. [1]

1.1.1. The function of succinate dehydrogenase and the role of succinate

The Krebs cycle consists of chain of chemical reactions in order to generate energy for cells. The whole CAC takes place in the mitochondrial matrix. It uses carbohydrates, fats, amino acids and proteins to oxidase acetyl-coenzyme A (acetyl CoA). The main source of acetyl CoA comes from the glycolysis, but can be derived from fatty acid oxidation as well. The intermediates of the CAC serve as substrate for biosynthetic pathways.

The CAC result in a total of four molecules of ATP, ten molecules of NADH, and two molecules of $FADH_2$. Electrons from NADH and $FADH_2$ are then transferred to molecular oxygen through the oxidative phosphorylation. [2]

Succinate dehydrogenase catalyzes the 7^{th} step of the CAC (tricarboxylic acid cycle or Krebs cycle). It catalyzes the oxidation of succinate to fumarate along with the reduction of ubiquinone (Coenzyme Q) to ubiquinole, by transferring electrons thru FAD-$FADH_2$ (Figure 1).

Succinate dehydrogenase or complex II is also involved in the oxidative phosphorylation (OXPHOS) or electron transport chain, representing the major source of cellular energy. The OXPHOS takes place in the inner membrane of the mitochondria and consists of four complexes; complex I, II, III and IV. The fifth complex is the ATP synthase, which uses the proton gradient to synthesize 32 to 34 ATP molecules. The flow of electrons from NADH and $FADH_2$ thru the protein complexes is associated with pumping protons to the

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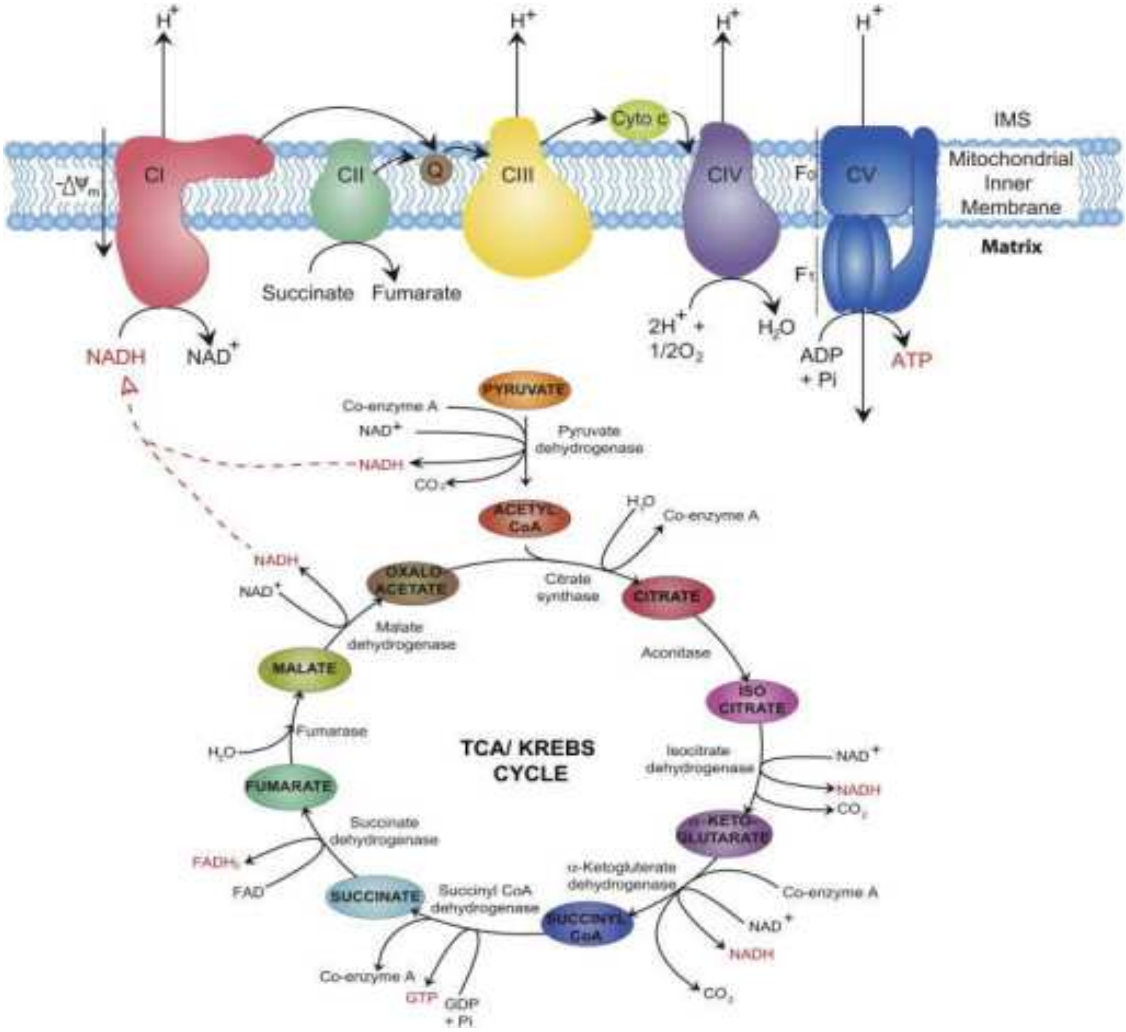
intermembrane space of the mitochondria, which results in a proton gradient and builds up the transmembrane potential. This is necessary for driving complex V to synthetize ATP. However, complex II is not coupled with a proton pump and transfers electrons to ubiquinone without contributing to the proton gradient. [3]

Figure 1. Citric acetate cycle and oxidative phosphorylation.



Succinate dehydrogenase catalyzes succinate to fumarate oxidation in citric acid cycle and as complex II participates in the electron transfer in the oxidative phosphorylation.

Based on work Osellame LD, Blacker TS DM. Cellular and molecular mechanisms of mitochondrial function. *Best Pr Res Clin Endocrinol Metab*. 2012;26(6):711–723.

The substrate of SDH enzyme, succinate, is a distant product of the α-ketoglutarate dehydrogenase complex. It is involved in several metabolic pathways including a macrophage-specific metabolic pathway generating itaconate [4], it is connected with the metabolism of branched-chain amino acids, heme synthesis, ketone bodies utilization and the GABA shunt. [5,6 7]

Regarding tumorigenesis, succinate is considered as a critical mediator of the hypoxic response, and it has also been suggested that SDH plays an important role in ROS homeostasis of cells producing superoxide and H_2O_2. [8]

Succinate was also involved in posttranslational protein modification called succinylation by this mechanism succinate might be involved in stabilization of certain proteins. In specific tumours it was demonstrated that succinate stabilizes hypoxia inducible factor 1α, a key transcription factor for regulating molecules which are involved in adaptation to hypoxia and in facilitating blood vessel genesis (vascular endothelial growth factor, platelet growth factor etc.). [9]

In addition, succinate was discovered to exert its effects outside of cells in para- and autocrine manners, mediated by the expression of at least one plasmalemmal succinate receptor type. [10]

Based on these fundamental processes it is not surprising that mutations of genes encoding the subunits of SDH complex have been implicated in the pathogenesis of various diseases including oxidative stress, tumour formation, neurodegeneration, hypoxia or “just simple energy deficiency”.

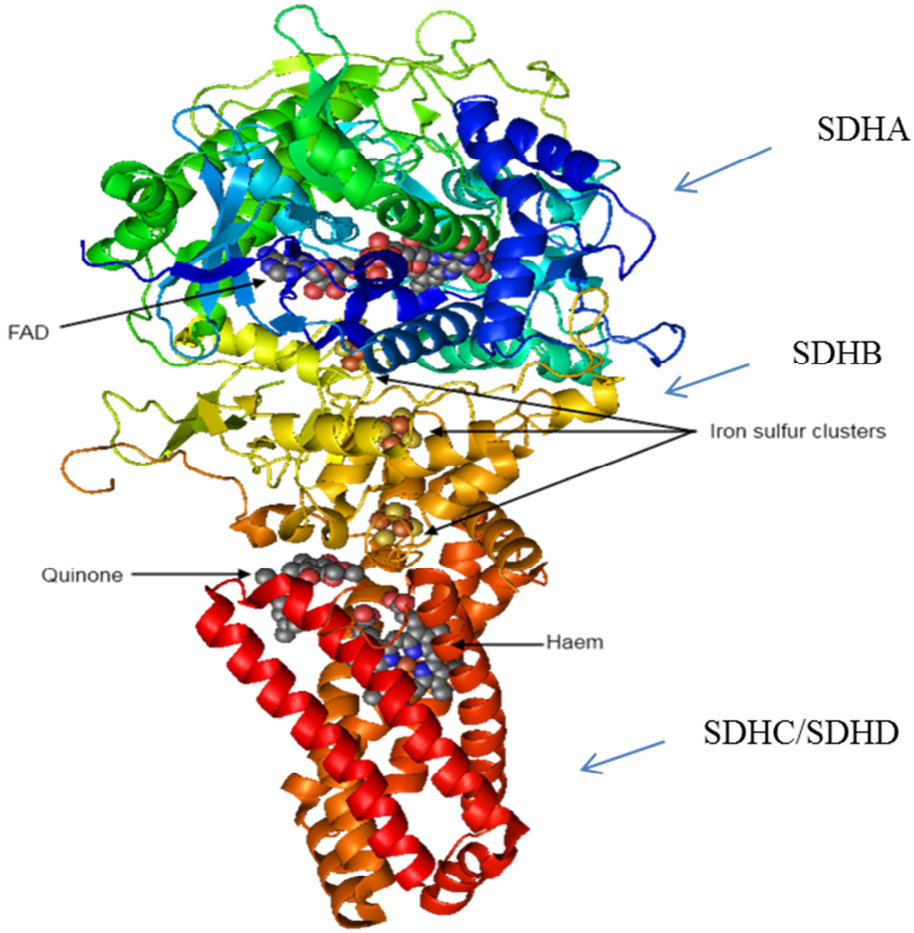
1.1.2. The structure of SDH

SDH consists of a hydrophilic head that protrudes into the matrix compartment and a hydrophobic tail that is embedded within the IM with a short segment projecting into the soluble intermembrane space. The hydrophilic head consists SDHA (flavoprotein) and SDHB (iron sulphur protein), forming the catalytic core. Here are the binding sites for FAD

cofactor and succinate. Three iron-sulphur clusters can be found in the SDHB subunit and these clusters mediate electron transfer to ubiquinone (Figure 2).

The hydrophobic tail consists of SDHC and SDHD subunits. The enzyme complex binds to membrane through these subunits. The structure of the enzyme complex is constructed of six transmembrane helices containing one heme b group and a ubiquinone-binding site. [11] (Figure 2)

Figure 2. Structure of succinate dehydrogenase.



Succinate dehydrogenase [CC-BY-SA-3.0 Steve Cook, based on PDB 1NEK] The 'top' of the enzyme pokes into the mitochondrial matrix and oxidises succinate; the 'bottom' of the enzyme is dissolved in the lipid of inner mitochondrial membrane, and reduces ubiquinone.

1.1.3. Chromosomal localization of genes encoding the SDH subunits

All four subunits of SDH or complex II are encoded by genes located in the nuclear genome. *SDHA* encoding gene is mapped to the p arm of chromosome 5 at locus 15, *SDHB* gene is localized on the p arm of chromosome 1 at locus 36. *SDHC* gene is encoded on the q arm of chromosome 1 at locus 23. *SDHD* and *SDHAF2* genes are encoded on the q arm of chromosome 11 at locus 23.1 and 13, respectively. Number of exons and number of amino acid residues are included in Table 1.

Genetic mutations of these genes are associated with familial paraganglioma syndrome, childhood T-cell acute leukaemia and gastric stromal tumours. [12–16]

Table 1. Chromosomal localization of succinate dehydrogenase subunits.

Gene	Ensemble ID	Chromosomal localization	Number of exons	Number of amino acids
SDHA	ENSG00000073578	5p15	15	644
SDHB	ENSG00000117118	1p36	8	280
SDHC	ENSG00000143252	1q23	6	169
SDHD	ENSG00000204370	11q23.1	4	159
SDHAF2	ENSG00000167985	11q13	4	166

SDHA - succinate dehydrogenase subunit A, SDHB - succinate dehydrogenase subunit B, SDHC - succinate dehydrogenase subunit C, SDHD - succinate dehydrogenase subunit D, SDHAF2 – succinate dehydrogenase complex assembly factor 2

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1.2. Pheochromocytoma and paraganglioma

1.2.1. Definition, anatomical distribution

The term pheochromocytoma means “dusky-colored tumour” and it was historically derived from the color change that occurs when the tumour tissue was immersed in chromate salts. [17] Pheochromocytomas are rare catecholamine-producing tumours arising from neural crest-derived chromaffin cells in the adrenal gland. Chromaffin cells are also found in the sympathetic ganglions; sympathetic extra-adrenal paragangliomas are generally confined to the lower mediastinum, abdomen, and pelvis (e.g. the aortic chemoreceptors and the Zuckerkandl-organ), and are typically hormone secreting. In contrast, parasympathetic paragangliomas are located predominantly on the skull base, neck, and upper mediastinum (e.g. carotid artery/body). [18,19] [20] (Figure 3)

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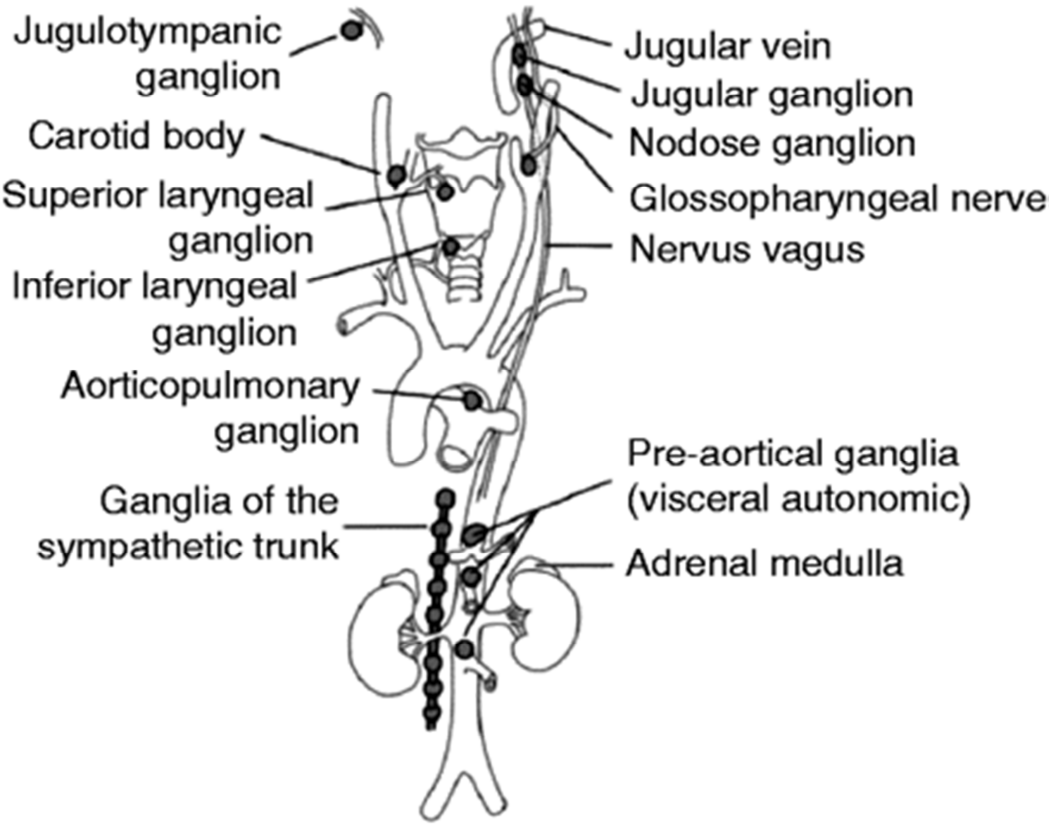
Figure 3. Anatomical distribution of pheochromocytoma and paragangliomas. [21]



Based on Lips C, Lentjes E, Höppener J, Luijt R van der, Moll F. Familial paragangliomas. *Hered Cancer Clin Pract*. 2006. doi:10.1186/1897-4287-4-4-169.

The prevalence of this tumour is estimated between 1:6500 to 1:2500 in the United States [22] but the autopsy reports suggest a prevalence of 1:2000 and suggest that many of these tumours remain undiagnosed. [23,24] An increased frequency is noted in people subjected to chronic hypoxia, living at higher-altitude regions or in the presence of respiratory or heart diseases. [25] The incidence of pheochromocytoma is 2 to 8 per million persons per year. [26] Other tumours, as head-and-neck, abdominal, and pelvic PGL have an incidence of 0.5 per million per year. [27] Paragangliomas in the Zuckerkandl-organ are the most common sympathetic and carotid body tumours are the most common parasympathetic extra adrenal paragangliomas. [28]

Pheochromocytoma and paraganglioma can occur at all ages, but have a peek incidence at the 4th and 5th decade, with an almost equal distribution between men and women. [29] [30] Pheochromocytoma is present in 0.1% to 1% of patients with hypertension [31,32] and it is present in approximately 5% of patients with incidentally discovered adrenal masses. [33] About 90% of PHEOs are unilateral, but bilateral tumours are seen in higher proportion in syndromic cases. [27]

The risk for malignant transformation is greater for extra-adrenal sympathetic paragangliomas than for pheochromocytomas or skull base and neck paragangliomas. [20,25] It is difficult to determine the malignancy of PHEOs and PGLs as only the metastasis to lymph node, bone, liver, or lung confirms malignancy. [19,34] However, pathological criteria, as size, weight, presence of tumour necrosis, a greater than 4% of Ki-67 index and the absence of S100 by immunohistochemistry have been shown to be associated with malignancy. [35]

Early diagnosis and resection of the tumour can cure most of the cases. The diagnosis is difficult because clinical features/symptoms can mimic other diseases or can be very unspecific or uncharacteristic.

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1.2.2. Clinical features

Sympathetic paragangliomas secrete catecholamines, mainly adrenalin and noradrenalin; parasympathetic paragangliomas are most often (ca. 95%) hormonally silent or have low catecholamine production. Symptoms of PGL/PCC result either from the mass effects or catecholamine hypersecretion. The main symptoms related to hormone hypersecretion are the following:

- Hypertension (paroxysmal or sustained)
- Palpitation and tachycardia
- Sweating attacks
- Headache
- Facial flushing
- Chest and abdominal pain
- Anxiety and panic attacks
- Nausea
- Tremor
- Pallor
- Elevated fasting plasma glucose concentration

The symptoms are usually paroxysmal, but 50-60% of the patients have high blood pressure between the episodes. [36] In some cases the symptoms are more severe and cause cardiovascular and neurological manifestations. [37]

Parasympathetic paragangliomas show a slow-growing, painless mass and patients develop symptoms due to pressure of surrounding tissue or nerves: chocking, hoarseness, tickling cough, Horner's syndrome due to interruption of nervous tissue. [38]

1.2.3. Etiology/Genetic background and associated hereditary syndromes

Pheochromocytomas and paragangliomas are mainly sporadic tumours, formerly about 10% of all tumours associated with hereditary syndromes, including multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau syndrome (VHL) and neurofibromatosis type 1 (NF1). [39] A small percent of PHEO/PGL associated with Carney-triad, Carney-Stratakis syndrome and more rarely with MEN type 1. In the last decade several genes were discovered as genetic causes of pheochromocytoma and paraganglioma, making the prevalence of hereditary PHEO/PGL 30%–35% of all cases.

The common feature of hereditary syndromes is that they show an autosomal dominant inheritance, meaning that the affected individual receives one mutant gene from one of his/her parents. In some cases a de novo mutation in the germline of the patient may occur.

In sporadic (no germline disease-causing mutation) cases somatic, inactivating mutations can also be identified. The most common genes and the associated syndromes are summarized below.

1.2.3.1. Familial paraganglioma syndrome

Familial paraganglioma (FPGL) syndrome is caused by the germline heterozygous mutations of the *SDHx* genes (*SDHB, SDHC, SDHD*, encoding subunits B, C and D, respectively) [12,15,40] and the newly identified *SDH5* gene. [16] *SDHx* genes encode subunits of the mitochondrial complex II (succinate dehydrogenase, SDH), an enzyme involved in oxidative phosphorylation and intracellular oxygen sensing and signalling.

Some differences between the mutation types have been observed. Most of the *SDHB* and *SDHC* mutations are mutations which result in truncated protein or leading to amino acid change of the iron sulphur clusters in the SDHB, while mutations of the *SDHD* gene are more likely nonsense mutations and deletions/insertions. The genotype-phenotype associations have been summarized in Table 2.

Biallelic mutation of the *SDHA* leads to Leigh syndrome, characterized by mitochondrial encephalopathy and optic atrophy. In 2010 the first *SDHA* gene mutation was described

associating with abdominal PGL and head and neck PGL. [41] This latter finding added *SDHA* to genes representing genetic susceptibility for PHEO/PGL.

SDHB mutations cause hereditary paraganglioma syndrome type 4. *SDHB*-related PGLs are associated with abdominal, pelvic tumours, and show single presentation in two third of the cases. Mutations in the *SDHB* gene are associated with a high risk of developing metastases [29] and about 20%–30% of patients already have metastatic disease at the time of the initial diagnosis [42], while mean age at onset is typically 25-30 years. [43] The diagnosis of *SDHB*-related PHEOs/PGLs is often delayed, most likely because of the less typical catecholamine excess-related clinical presentations compared with other apparently sporadic or hereditary PHEOs/PGLs. This is partially due to the fact that these tumours can have either a biochemically silent phenotype, a low intratumoural catecholamine content, or a purely dopaminergic phenotype. [42,44]

SDHC gene mutations cause hereditary paraganglioma syndrome type 3 with autosomal dominant inheritance. Classical clinical presentation for these tumours is solitary head-neck paraganglioma with a low risk of malignancy. The mean age at diagnosis is 38 years. [45]

Mutations of the *SDHD* gene are causing hereditary paraganglioma syndrome type 1. Its clinical presentation shows multiple abdominal and head-neck paragangliomas with an age of onset at 28-31 years. [47, 48] *SDHD*-related PHEOs/PGLs, especially those derived from the parasympathetic nervous system of the head and neck, are much less aggressive than *SDHB*-related PGLs. It has to be mentioned that SDHD gene is maternally imprinted, meaning that disease-causing mutations are inherited exclusively from the paternal side.

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Table 2. Familial paraganglioma syndrome genotype-phenotype association.

Syndrome	Chromosomal localisation Gene	Phenotype
PGL1	mutations on chromosome 11 (11q23) SDHD	head and neck, intraabdominal paragangliomas pheochromocytomas
PGL2	11q13 (SDHAF2, SDH5)	paraganglioma
PGL3	1q21-23 (SDHC)	paraganglioma
PGL4	1p36 (SDHB)	head and neck paragangliomas pheochromocytomas

PGL - Familial paraganglioma syndrome, SDHAF2 – succinate dehydrogenase complex assembly factor 2, SDHB - succinate dehydrogenase subunit B, SDHC - succinate dehydrogenase subunit C, SDHD - succinate dehydrogenase subunit D

1.2.3.2. Multiple endocrine neoplasia type 2A and 2B

The *RET* (rearranged during transfection) proto-oncogene located on chromosomal region 10q11.2 consists of 21 exons encodes a receptor tyrosine kinase. [48] Germline, gain of function mutations (mainly missense) of the *RET* proto-oncogene cause multiple endocrine neoplasia type 2 (MEN2), an autosomal dominantly inherited disease with an approximate prevalence of 2.5 per 100.000 in the general population. [48] The gene mutation are found in specific, hot spot regions (exons: 10, 11, 13, 14, 15 and 16) of the RET proto-oncogene. [49] MEN2 has three subtypes: i) MEN2A, characterized by medullary thyroid carcinoma (MTC), pheochromocytoma (PHEO) and primary hyperparathyroidism; ii) MEN2B, which presents with the most aggressive MTC, pheochromocytoma, neuromas and marfanoid phenotype; and iii) familial MTC (FMTC), the mildest form of MTC. (Table 3, Table 4)
Pheochromocytoma occurs in approximately 50% of the MEN2 patients. They are bilateral in most of the cases and have a low rate for malignancy. [50,51] Usually, pheochromocytoma is diagnosed at the same time as the medullary thyroid cancer, [52,53] while some more recent studies showed that PHEO was diagnosed after the diagnosis of MTC [54,55], and very rarely PHEO precedes MTC.
Analysis of the *RET* gene showed that missense mutations in codon 634 in exon 1 were the most frequent mutations (mutation hot-spot) and the TGC634CGC (Cys-Arg) mutation frequently associated with PHEO. [45,53,56]

Table 3. MEN2 syndromes and associated phenotypes.

MEN Type	Clinical manifestation	Genotype
MEN2A	MTC PHEO primer hyperparathyreosis	mutation s in exon 10 and 11, codon 634 is a mutation hotspot
MEN2B	MTC (aggressive form) PHEO marfanoid habitus mucosal neuromas	Codon 918 in exon16
FMTC	MTC (mild form)	Exons: 10,11 or 5, 8, 13-16; codons: 609, 611, 618, 620 or 790, 791, 768, 804

MEN - multiple endocrine neoplasia, MTC - medullary thyroid cancer, PHEO - pheochromocytoma, FMTC - familial medullary thyroid cancer.

Table 4. The effect of *RET* mutations on the aggressiveness of MTC. [57]

Risk of MTC development	Mutation in codon
1. Most aggressive, develops in infancy	883, 918, 922
2. Aggressive, develops in childhood	611, 618, 620, 634
3. Less aggressive, develops in older age	609, 768, 790, 791, 804, 891

RET – rearranged during transfection, MTC – medullary thyroid cancer

Based on Brandi ML, Gagel R, Angeli A, Bilezikian J, Beck-Peccoz P, Bordi C, Conte-Devolx B, Falchetti A, Gheri R, Libroia A, Lips C, Lombardi G, Manelli M, Pacini F, Ponder B, Raue F, Skogseid B, Tamburrano G, Thakker R, Thompson N, Tomasetti P, Tonelli F, Wells MS. Guidelines for diagnosis and therapy of MEN type 1 and type 2. *J Clin Endocrinol Metab*. 2001;86:5658–5671.

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1.2.3.3. von Hippel-Lindau syndrome (VHL)

The *VHL* tumour suppressor gene is located on the 3rd chromosome (3p25-26), mutation or deletion of the gene causes the von Hippel-Lindau syndrome (VHL-syndrome). The disease is autosomal dominantly inherited. Pheochromocytoma, retina angioma, cerebral haemangioblastoma, renal carcinoma, cyst of the kidney and pancreas are the main manifestations. In 7-20% of the cases patients develop pheochromocytoma and these tumours are mainly asymptomatic and affect both adrenals. VHL syndrome is classified based on the appearance or lack of pheochromocytoma in subtypes 1, 2A, 2B and 2C (Table 5).

VHL mutations have the highest predominance in paediatric cases and show the highest prevalence for second contra lateral tumours. Therefore, these patients need close follow-up, usually in every 1-3 years after the first diagnosis. [45 58]

Table 5. Classification of VHL syndrome.

Type	Manifestation
Type 1.	no PHEO
Type 2.	Risk of PHEO
Type 2A	kidney cancer, low risk of PHEO
Type 2B	kidney cancer, high risk of PHEO
Type 2C	only PHEO

VHL - von Hippel-Lindau syndrome, PHEO - pheochromocytoma

1.2.3.4. Neurofibromatosis 1

Neurofibromatosis type 1 (von Recklinghausen disease) is characterized by skin lesions (café-au-lait spots), growth of tumours along the nerve in the skin (neurofibromas) and nodules in the iris (Lisch-nodules). Patients with NF1 syndrome have an increased risk for developing optic glioma, leukemia and gastrointestinal tumours. The frequency of NF1 is about 1:3000 to 1:4000 and therefore is one of the most frequent autosomal dominant tumour syndrome, however about 50% of the cases result from new mutations. Pheochromocytoma appears in 1% - 5.7% of all cases, although 50% of the patients with high blood pressure have pheochromocytoma. [59]

Inactivating, somatic mutations of *NF1* were described in 41% of sporadic PHEO where LOH at the *NF1* locus were also described [60]**,** suggesting that lack of function contributes to the pathogenesis of PHEO.

The *NF1* gene is located on chromosome 17 (17q11.2), the NF1 protein participates in the regulation of the ras oncogene and its function resembles to tumour suppressor function.

Patients with NF1 syndrome are suggested clinical and laboratory surveillances for PHEO every 3 years. [61]

1.2.3.5. Recently identified, novel genes causing PHEO/PGL

*TMEM127 (*2q11) was identified in 2010 as a new pheochromocytoma susceptibility gene. [62] The encoded protein is a transmembrane protein which is involved in the mammalian target of rapamycin signalling pathway. [46] The *TMEM127* associated syndrome is autosomal dominantly inherited. Clinical presentation is characterised by a presence of a mainly unilateral PHEO in patients with no prior family history, however a recent study showed that bilateral, extra-adrenal PGL cases were also described. [63,64]

Pathogenic *MAX* (myc-associated factor X) (14q23) gene variants were shown to predispose to PHEO/PGL. [65] MAX is a transcription factor involved in cellular

proliferation, differentiation and apoptosis. It seems that the phenotype associated with *MAX* mutation shows the appearance of early onset (age <30 years), bilateral PHEO. [66]

KIF1B was reported in a single case to be responsible for PHEO. This gene is frequently implicated in inherited and sporadic neural crest tumours such as neuroblastomas. [20,67]

EGLN1 (formerly known as *PHD2*), *IDH1, HIF2A* and *FH* genes have been reported to be associated with hereditary PGL/PHEO, but their clinical significance is still unclear (Table 6). [68,69]

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Table 6. Hereditary syndromes and associated genes with year of identification.

Syndromes	Gene	Year of identification
Neurofibromatosis type 1	*NF1*	1990
von Hippel-Lindau	*VHL*	1993
multiple endocrine neoplasia type 2	*RET*	1994
PGL1	*SDHD*	2000
PGL4	*SDHB*	2000
PGL3	*SDHC*	2001
PHEO, neuroblastoma, lung cancer	*KIF1B beta*	2008
PGL, erythrocytosis	*EGLN1/PHD2*	2008
PGL2	*SDHAF2*	2010
PHEO, PGL	*TMEM127*	2010
PHEO, PGL	*SDHA*	2011
PHEO, PGL	*MAX*	2011
PHEO	*FH*	2014
PHEO	*MDH2*	2015

PHEO – pheochromocytoma, PGL – paraganglioma, NF1 - neurofibromatosis type 1, VHL-von Hippel-Lindau syndrome, RET – rearranged during transfection, SDHAF2 – succinate dehydrogenase complex assembly factor 2, SDHA - succinate dehydrogenase subunit A, SDHB - succinate dehydrogenase subunit B, SDHC - succinate dehydrogenase subunit C, SDHD - succinate dehydrogenase subunit D, KIF1B – kinesin family member 1B, EGLN1 – prolyl hydroxylase domain-containing protein 2, TMEM127 – transmembrane protein 127 , MAX – myc-associated factor X, FH – fumarate hydratase, MDH2 – malate dehydrogenase.

1.2.4. Possible genetic modifiers

Germline gain of function mutations of the *RET* proto-oncogene cause MEN2. Several important genotype–phenotype associations have been determined; the most commonly affected codon, the codon 634 (nearly 85% of MEN2A cases), frequently associates with PHEO and hyperparathyroidism, whereas mutations of codons 609, 611, 618, and 620 (accounting for 10–15% of MEN2A) usually associate with the milder form of MEN2. [70,53,56]

However, the phenotypic heterogeneity observed even in members of the same family suggests that other factors, for example genetic modifiers, may influence the clinical manifestation of the disease. [71–74] As summarized earlier, mutations of *SDHx* genes are causing PHEO/PGL syndromes. [75] Because both MTC and PHEO/PGL arise from neural crest-derived precursor cells it may be hypothesised that same genetic factors may be involved in both tumour types. Accumulation of amino-acid coding polymorphisms (S163P in *SDHB*, G12S, and H50R in *SDHD*) have been found among patients with MTC, especially in those with familial tumours. [76] In addition, these rare genetic variants have been identified in patients with Cowden-like syndrome [77] and the H50R polymorphism has been described in six members of a family with non-RET-associated C-cell hyperplasia and hypercalcitoninemia. [78] These previous data may suggest a possible connection between *SDHx* polymorphisms and familial MTC and/or C-cell hyperplasia/hypercalcitoninemia.

1.2.5. Biochemical characteristics

The diagnosis of *SDHB*-related PHEOs/PGLs is often delayed, most likely because of the less typical catecholamine excess-related clinical presentations compared with other apparently sporadic or hereditary PHEOs/PGLs. This is partially due to the fact that these tumours can have either a biochemically silent phenotype, a low intratumoural catecholamine content, or a purely dopaminergic phenotype. [42,44] In contrast, *SDHD*-related

PHEOs/PGLs, especially those derived from the parasympathetic nervous system of the head and neck, are much less aggressive. The presence of *SDH* mutations impairs oxidative phosphorylation and the Krebs cycle, resulting in metabolic abnormalities, including succinate accumulation. [79]

The Warburg effect

The Warburg effect is the 7th hallmark of most cancer types, along with persistent growth signals, evasion of apoptosis, angiogenesis, insensitivity to anti-growth signals, unlimited replication potential, invasion and metastasis. [80]

In 1956 Dr. Otto Warburg described the effect that tumour cells show a high glucose uptake in the presence of oxygen accompanied by lactic acid production, aerobic glycolysis. [81] Dr. Warburg's suspect that functional defect in the mitochondria causes impaired respiration [82] has been proved in the past years. The increasing knowledge of the Warburg effect lead us to new treatment [83] and diagnostic approaches, e.g. 2-^{18}F-fluoro-2-deoxy-D-glucose position emission tomography. The aerobic glycolysis yields only 2 ATP molecules, but/and the tumour cells show an increased glycolysis.

Mutations in *SDH* genes lead to loss of function in SDH enzyme, which then lead to succinate accumulation. Succinate inhibits prolyl hydroxylases (PHD), which has a role to modify and degrade hypoxia inducible factor 1α (HIF1α). Increasing the levels of HIF1α triggers tumourigenesis [84 85 79].

Presently, the ultimate diagnosis of these tumours is based on immunohistochemistry to detect the presence or absence of the SDHB protein or genetic testing for an *SDH* mutation or deletion [86,87]. Although next-generation sequencing methods will significantly reduce the costs of such testing, currently this genetic testing is still costly and therefore limited or even unavailable in many countries. Neither method can be used e.g., to predict therapeutic responses of these tumours, their resistance to various therapies, for follow-up after a therapy is completed, or to assess their progression over time.

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1.2.6. Diagnosis

The diagnosis of hereditary pheochromocytomas and paragangliomas is complex, meaning physical examination, family history, biochemical and molecular genetic testing and imaging studies.

Why is it important to suspect PHEO or PGL?

The consequences of catecholamine hypersecretion can lead to serious lesions or even death. As 25-30% of the sporadic cases are caused by a germline mutation, family screening can help to diagnose and treat the tumour earlier.

Malignancy is defined by existence of metastases; and patients with mutation in the *SDHB* gene have a high risk for metastasis.

1.2.6.1. Physical examination and family history

A detailed family history and personal medical history is very important in cases with PHEO/PGL. Personal medical history should cover the following: symptoms of catecholamine excess, paroxysmal symptoms that may be triggered and enlarging masses.

1.2.6.2. Biochemical testing

In the diagnosis of PHEO and PGL we can use the biochemical characteristics of the tumour, by measuring the levels of the secreted hormones and their major metabolites. Plasma free and fractionated metanephrines are the first test to perform when PHEO/PGL is suspected. (Table 7)

Table 7. Diagnositic sensitivity of plasma and urinary catecholamines and their metabolites in hereditary and sporadic pheochromocytoma.

	Sensitivity		**Specificity**	
	Hereditary	Sporadic	Hereditary	Sporadic
Plasma				
Catecholamine	69%	92%	89%	72%
Metanephrine and Normetanephrine	97%	99%	96%	82%
Urine				
Fractionated metanephrines	96%	97%	82%	45%
Catecholamines	79%	91%	96%	75%

Based on Lenders JW, Pacak K, Walther MM, et al. Biochemical diagnosis of pheochromocytoma: which test is best? *JAMA*. 2002;287:1427–1434.

Urine metanephrines have superiority over free catecholamines and vanillylmandelic acid (the end product of catecholamine metabolism). However the diagnostic sensitivity of plasma free metanephrine and normetanephrine are superior [88] and their diagnostic accuracy has been confirmed. Unfortunately, determination of plasma metanephrines and normetanephrin or plasma free catecholamines are not routinely available in many countries.

Liquid chromatography followed by mass spectrometric or electrochemical detection methods are suggested to use for measuring metanephrines and catecholamines. Patients/specimens should be referred to specialist centres.

Blood sampling for plasma metanephrines should be done after 30 minutes of supine rest. [89] This is usually hard to carry out at clinical centres but doing blood sampling without supine position will lead to an increase in false-positive results. In these cases it is recommended to measure urine fractionated metanephrines. However, false-positive results have a 19-21% rate in plasma free and urine fractionated metanephrines. [90]

In 50% of the patients with pheochromocytoma, both normetanephrine and metanephrine are elevated; by at least 3 fold or more above the upper cut-off.
The clonidine test is recommended to distinguish the false-positive cases from the true-positive ones.
Some medications can interfere with these measurements or with the catecholamine disposition, not to mention physiological stress with severe conditions.
All positive cases should be followed up, and sometimes second, confirmatory determinations are required.

1.2.6.3. Imaging studies

Imaging studies are recommended to locate the tumour in patients with positive biochemical tests. However, only imaging studies can identify/locate the tumour in patients with biochemically negative results.
CT is the first choice imaging modality for the thorax, abdomen and pelvis, its sensitivity is 88-100%. [91] Tumours greater than 5mm can already be detected by CT.
MRI has a better sensitivity in patients with extra-adrenal, recurrent and metastatic tumours and head and neck paragangliomas. [91–93] MRI is preferred in children, pregnant women and has a sensitivity of almost 100%. Both methods are used for tumour staging as well. [91,94]
^{123}I-metaiodobenzylguanidine (^{123}I-MIBG) scintigraphy is a functional imaging modality in metastatic cases and is used when radiotherapy with ^{131}I-MIBG is planned.
In *SDHx*-related tumours 2-^{18}F-fluoro-2-deoxy-D-glucose positron emission tomography (^{18}F-FDG-PET) has superiority to other imaging techniques. [94]

1.2.6.4. Molecular genetic testing

Due to the large number of genes responsible for the development of PHEO/PGL the genetic testing remains a diagnostic challenge. Since 1990, 14 different susceptibility genes have been reported. Both laboratory workload and cost of testing of all genes are still significant despite of the lower price of molecular biological reagents. Phenotype oriented

guidelines allow us some priorization in the order of genes tested but after a negative result the remaining genes should also be examined. Therefore, it would be ideal that after exclusion of some syndrome-associated genes based on the obvious phenotype features (i.e. because of typical manifestation the NF1 gene is rarely tested) all of the remaining genes would be tested at the same time. Recent technical improvements in sequencing technology, the next generation sequencing (NGS) platforms - allow us to use whole exome or targeted resequencing of all these genes. [95] The usefulness of NGS has been demonstrated not only in resequencing of already known genes, but also in discoveries of novel genes associated with PHEO/PGL. Confirmation of results and a negative NGS result does not exclude the possibility of mutations especially the presence of large deletions. Therefore, the gold standard methodology for identification of pathogenic mutation is the PCR amplification of the coding region of target genes followed by Sanger sequencing. For large deletion analysis multiple ligation probe amplification (MLPA) should be also performed. In addition, the Endocrine Society clinical practice guideline recommend the use of a clinical feature-driven diagnostic algorithm to establish the priorities for specific genetic testing in PHEO/PGL patients with suspected germline mutations delivered within the framework of health-care. [91,96]

1.2.7. Treatment of pheochromocytoma and paraganglioma

Surgery is the definitive treatment of PHEO and PGL, if the tumour location allows resection. To prevent cardiovascular complications patients with hormonally functional PHEO/PGL should receive preoperative blockade, the first choice should be α-adrenergic receptor blockers. Calcium channel blockers are the most common add on drugs and β-adrenergic receptor blockers are used in co-administration to control tachycardia. These latter two drugs are not recommended to us in single medication. α-adrenergic receptor blocker treatment should be administered at least 7 days before surgery. [97]

For surgery, recommendations suggest minimally invasive techniques. Laparoscopic adrenalectomy is the first choice, but invasive tumours or tumours with size over 6 cm are

recommended for open resection. Paragangliomas are suggest for open resection, although in some cases (e.g.: small, non-invasive, location) laparoscopic resection can be done.

After surgery patient personalized follow up is necessary depending on the genetic results. In syndromic cases, where often bilateral tumours develop, a minimal invasive tumourectomy (adrenal sparing surgery) with left adrenal cortex tissue is advised. [98]

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2. OBJECTIVES

During my PhD training I aimed to collect and to summarize the evidence of the role of *SDHx* variants in the pathogenesis of PHEO/PGL. My specific aims were:

2.1. to evaluate the role of Mutations of SDHx genes in Hungarian patients with PHEO/PGL

- *to determine the prevalence of germline mutations in the SDHx, SDHAF2, MAX and TMEM127 genes in Hungarian patients with apparently sporadic PHEO/PGL.*
  to describe the detailed phenotype of the first Hungarian case with SDHD gene mutation.
- *to collect and to report the genotype-phenotype association in patients with PHEO/PG.*
- *to identify novel mutations among Hungarian patients with Pheo/PGL*

2.2. to test whether polymorphisms of *SDHx* genes are phenotype modifiers in patients with MEN2A syndrome, therefore I aimed to

- *to determine the prevalence of SDHx polymorphisms in patients with RET mutations (MEN2 patietns), in patients with sporadic medullary thyroid cancer (MTC), sporadic PHEO, healthy subjects*

2.3. to identify the metabolic consequences of *SDHx* mutations/deletions in tumour tissues and cell lines. In order to fulfil this aim I aimed:

- *to measure the levels of the two Krebs cycle metabolites, succinate and fumarate, in tumour tissue and in human plasma samples obtained from patients with Pheo/PGL*
- *to determine the succinate to fumarate ratio in mouse pheochromocytoma (MPC) and mouse tumour tissue (MTT) cells*
- *to test whether this difference propose the implementation of succinate/fumarate measurements in clinical diagnosis.*

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3. METHODS

3.1. Germline mutation prevalence in Hungarian patients with pheochromocytoma and/or paraganglioma

3.1.1. Patients

Our database containing the clinical and laboratory data of 129 patients diagnosed and followed up at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University with clinical diagnosis of PHEO/PGL between 1998 and 2014 was reviewed in order to select cases for comprehensive genetic testing. All patients underwent genetic counselling and written informed consent was obtained before genetic analysis.

Of these patients, the clinical diagnosis was confirmed by pathological examination of the surgically removed tumour tissues in 92 cases. Mutation screening of the *RET* and *VHL* genes identified 4 *RET* mutation carriers and 4 patients with germline *VHL* mutations. [99–101] In two cases the specific phenotype features indicated neurofibromatosis type 1. These patients were excluded from this current analysis and *SDHB, SDHC, SDHD, SDHAF2, MAX* and *TMEM127* mutation analysis was performed in 82 cases. The main demographic and pathological data are summarized in Table 8.

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Table 8. Main genotype-phenotype associations in Hungarian patients with PHEO/PGL.

Cause of PHEO/PGL	Age (years)	Malignant/ recurrent	Bilateral or multiple locations
Genetic cause (n=11)	34,6 (19-51)	3/11 (27.2 %)	8/11 (72.7%)
SDHD (n=1)	32	0	1/1 (100 %)
SDHB (n=7)	31.4 (19-38)	3/7 (42.8%)	5/7 (71.4 %)
TMEM (n=3)	40 (22-51)	0/3 (0 %)	2/3 (66 %)
No genetic cause (n=71)	40,4 (13-78)	12/71 (16.9 %)	3/71 (4.2%)
Total (n=82)	38,8 (13-78)	15/82 (18.3%)	11/82 (13.4%)

PHEO – pheochromocytoma, PGL – paraganglioma, *SDHD* – Succinate dehydrogenase subunit D, *SDHB* - Succinate dehydrogenase subunit B, *TMEM127* – transmembrane protein 127

3.1.2. The first Hungarian case with extra-adrenal pheochromocytoma associated with SDHD gene mutation

In 2002 it became possible to analyse *SDH*x gene mutations at the 2nd Department of Medicine, Semmelweis University. In the beginning of my PhD work I identified the first disease causing mutation of the *SDHD* gene in a patient with extra-adrenal pheochromocytoma. This cases represents the first genetically confirmed case of hereditary paraganglioma/pheochromocytoma syndrome due to disease-causing mutation of the *SDHD* gene in Hungary. With this first case we have the opportunity to demonstrate maternal imprinting and to overview the pathomechanism in hereditary syndrome caused by *SDH* gene mutation.

3.1.3. Genetic testing of the RET, VHL, SDHB, SDHC, SDHD, SDHAF2, MAX and TMEM127 Genes Using Sanger Sequencing

After genetic counselling and obtaining informed consent of all 82 patients, underwent genetic testing for the *SDHB, SDHC, SDHD, SDHAF2, MAX* and *TMEM127* using conventional methods including PCR followed by Sanger sequencing. [99–101] Genomic DNA was extracted from peripheral blood using commercially available DNA extraction kits (DNA isolation from mammalian blood, Roche, or DNA isolation kit from blood, Qiagen LTD). Bidirectional DNA sequencing of all these genes and large deletion analysis of the *SDHB, SDHC* and *SDHD* genes were performed using multiplex ligation probe amplification. [101]

3.2. The G12S polymorphism of the *SDHD* gene as a phenotype modifier in patients with MEN2A syndrome

3.2.1. Patients

Written informed consent was obtained from all patients and family members who participated in the study. Patients underwent a complete clinical examination, laboratory testing, including serum basal calcitonin measurement [hCalcitonin IRMA kit (Diagnostic Systems Laboratories, Inc., Budapest, Hungary), reference range: male, < 15 pg/ml, female, < 10 pg/ml until December 2007; and Liaison (Diasorin SPA, Stillwater, MN, USA), reference value: male, < 18.9 pg/ml and female, < 6 pg/ml after January 2008], plasma parathyroid hormone (Elecsys; Roche Diagnostics, Basel, Switzerland), urinary catecholamine metabolites (high pressure liquid chromatography with electrochemical detection), and imaging studies, including cervical ultrasonography, thoracal and abdominal computed tomography (CT), and whole-body metaiodobenzylguanidine scintigraphy (MIBG).

3.2.1.1. Patients with MEN2 syndrome

In total, 77 patients with germline *RET* proto-oncogene mutations who were members of 21 unrelated families with MEN2 syndrome were identified by genetic screening at our centre. Of the 77 patients, 55 had MEN2A (mean age at diagnosis: 33.4±17 years; range: 7–76 years), three had MEN2B (mean age at diagnosis: 15.6±5 years; range: 10–20 years), and 19 had FMTC (mean age at diagnosis: 23.7±16.8 years; range: 2–57 years). The presence of PHEO and MTC were confirmed by histological examination of surgically removed tumours. Total thyroidectomy was performed in all patients with germline *RET* mutation in the symptomatic group and was also offered to all individuals from the asymptomatic group.

3.2.1.2. Patients with sporadic MTC

The study included 47 unrelated patients with histologically confirmed MTC evaluated consecutively at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998 and 2010. There were 15 men (age, mean ± SD, 44.7±13.3; range: 28–82 years) and 32 women (age, mean ± SD, 47.7±12,3; range: 23–76 years). Preoperative evaluation included medical history, physical examination, thyroid and abdominal ultrasonography, CT or magnetic resonance imaging (MRI), MIBG-scintigraphy, routine biochemical testing, serum calcitonin measurements, and mutation analysis of exons 10–14 of the RET gene.

3.2.1.3. Patients with apparently sporadic PHEO

The study included 48 unrelated patients with histologically confirmed sporadic adrenal pheochromocytomas evaluated consecutively at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University between 1998 and 2010. There were 16 men (age, mean ± SD, 36±14; range: 13–66 years) and 32 women (age, mean ± S.D, 42±14; range: 19–64 years). Pre-operative evaluation included medical history, physical examination, abdominal ultrasonography, CT or MRI, MIBG-scintigraphy, routine biochemical testing, and 24 h urinary catecholamine metabolite determination. The mutation analysis of *RET* exons 10–14 and the entire *VHL, SDHB*, and *SDHD* genes revealed no disease-causing mutations. Patients with confirmed *VHL* (five patients), *SDHB* (one patient), or *SDHD* (one patient) mutations were excluded from the study. Five patients were initially thought to have sporadic pheochromocytoma, but were later identified as having a disease-causing *RET* mutation and were included in the study as *RET* mutation carriers. MTC, either by elevated serum calcitonin or by postoperative histology, had been diagnosed in all of these patients. Genetic counselling and genetic screening for all first-degree relatives have been offered.

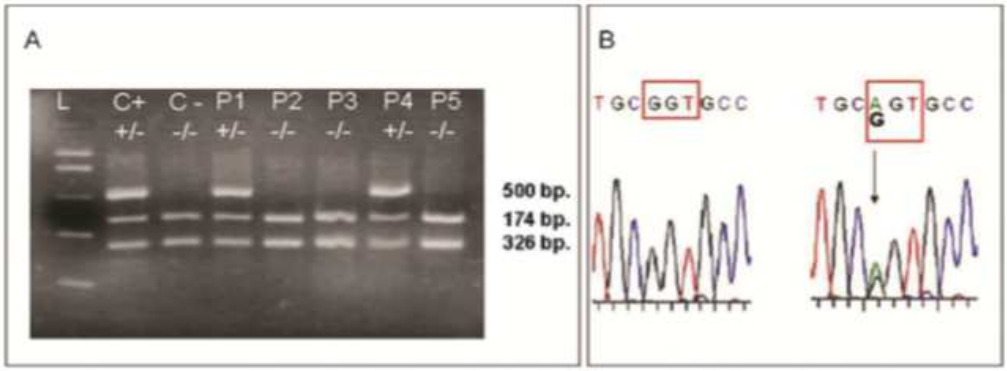
3.2.2. Germline mutation screening of the RET, VHL, SDHB, and SDHD genes

Genomic DNA was isolated from peripheral blood using the Roche DNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturers' instructions. *RET* proto-oncogene mutations were detected by direct sequencing as previously reported [99] [102]. Mutation analysis of the *VHL, SDHB*, and *SDHD* genes in cases of apparently sporadic PHEO were performed by direct sequencing of the entire coding region of the *VHL*, *SDHB*, and *SDHD* genes, as previously reported [99,101] , and large deletion analysis of the *VHL, SDHB, SDHC*, and *SDHD* genes performed using multiplex ligation probe amplification. [101]

3.2.3. Restriction fragment length polymorphism (RFLP) analysis for identification the G12S polymorphism of the SDHD gene

The nucleotide change of G to A, which corresponds to the G12S polymorphism, results in the preservation of the BanI restriction cleavage site. Therefore, digestion with the BanI restriction enzyme (New England BioLabs Inc., Ipswich, MA, USA) for 90 min at 37°C was performed after polymerase chain reaction (PCR) amplification of exon 1 of the *SDHD* gene for genotyping of *RET* mutation carriers, sporadic MTC patients, and 100 controls (Figure 4). Samples from patients with positive results were examined by direct DNA sequencing. The results obtained with both methods were the same in all cases.

Figure 4. Gel electrophoresis and chromatograms.



Panel A: Gel electrophoresis of PCR fragments after digestion with BanI for identification of the G12S polymorphism of the *SDHD* gene by RFLP (L = DNA ladder; C+ = positive control; heterozygote for G12S; C- = negative control; G12 normal, P1–P5 = patients. **Panel B:** Chromatograms of exon 1 of the *SDHD* gene showing the wild type and the heterozygote form of the G12S (GGT12AGT) polymorphism.

3.2.4. Statistical analysis

Baseline characteristics were compared using the chi-squared test or Fisher's exact test for qualitative variables, and Student's t test or Mann-Whitney's U test for quantitative variables. The statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used and $p<0.05$ was considered statistically significant.

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3.3. Biochemical consequences of *SDHx* mutations, succinate to fumarate ratio in *SDHB/D* associated paragangliomas

3.3.1. Materials

3.3.1.1. Human PHEOs/PGLs

PHEO/PGL tissue samples were collected at the National Institutes of Health (NIH) under clinical protocol 00-CH-0093, approved by the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD). Tissue samples were frozen in liquid nitrogen shortly after surgical removal of a tumour. All patients underwent genetic testing for known PHEO/PGL susceptibility genes except *SDHAF2* and neurofibromatosis 1 (*NF1*); the diagnosis of the latter was based on clinical grounds.

In the present study, we included four groups of tumours: *SDHB* (10 PGLs), *SDHD* (5 PGLs), apparently sporadic (6 PHEOs, 4 PGLs), and *NF1* (2 PHEOs). *NF1*-related PHEOs were included because of the genetic background of the mouse PHEO (MPC) and mouse tumour tissue (MTT) cells used in the *in vitro* experiments. A detailed summary of clinical and patient characteristics is described in Table 1.

3.3.1.2. Plasma samples

Patient blood samples were collected at the NIH under clinical protocol (00-CH-0093), approved by the Institutional Review Board of the NICHD. Blood samples were centrifuged at 3500 rpm at 4°C for 20 minutes, and the plasma was stored at - 80°C until further processing. In the present study we selected three samples for plasma measurements from each group (*SDHB*, *SDHD*, and apparently sporadic PHEOs/PGLs).

3.3.1.3. MPC and MTT cells

The MPC and MTT cell lines were used as described previously. [103,104] MTT cells are known to be more aggressive than MPC cells and show aggressiveness similar to human disease. [104] MPC and MTT cells were maintained at 21%O2, 5%CO2, 37°C in DMEM (4.5g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate; Life Technologies Corporation) supplemented with 10% fetal bovine serum (Gibco), 5%heat-inactivated horse serum (Gibco), and Anti-Anti 100_ (Penicillin/Streptomycin, Amphotericin B; Gibco). The medium was changed every 2 to 3 days and cells were passaged when 80%–90% confluence was reached. [103]

3.3.2. Silencing of SDHB in MPC and MTT cells

Early passages of MPC and MTT cells were transduced with lentiviral particles carrying either shRNA targeted against *mSDHB* or control shRNA (Thermo Fisher Scientific Inc). The cells were transduced at multiplicity of infection = 1 and maintained according to the manufacturer's instructions. Medium containing 1 µg/mL puromycin was used to select positive cells.
For the metabolic analysis we seeded 1.5 x 10^6 cells on a 6-cm dish. After 24 hours, cells were harvested in 1.5 mL PBS and snap-frozen in liquid nitrogen.

3.3.3. Western blotting

To evaluate the degree of *SDHB* silencing in MPC and MTT cells, Western blot analysis was performed. On 35-mm dishes, 1.0 x 10^6 cells were plated. The following day, they were lysed and the protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Thirty micrograms of total protein per well was loaded into a Criterion TGX

Precast Gel, 4%–12% (Bio-Rad Laboratories) and transferred to an Immobilon-P membrane (EMD Millipore Corporation). The membrane was blocked in 5% nonfat dry milk in 0.1% Tween in PBS for 1 hour. It was incubated with anti-SDHB antibody (Sigma-Aldrich Co) for 1 hour. β-Actin (Cell Signaling Technology Inc.) was used as a loading control. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), and blots were exposed to High Performance Chemiluminescence film (GE Healthcare) and analyzed with Image J 1.42q software (NIH).

3.3.4. Metabolic measurements

Procedures for the determination of succinate and fumarate have been described elsewhere [105] and are briefly related here. The organic acids were analyzed as their tertiary butyl dimethylsilyl ether derivatives using gas chromatography-mass spectrometry (GC-MS) in the electron impact mode and quantified using the 13C-labeled internal standards for each analyte. The *N*-methyl-*N*-(*tert*-butylmethylsilyl) trifluoroacetamide with 1% *tert*-butyldimethylchlorosilane reagent was purchased from Pierce Chemical Co, and the 13C-labeled organic acids were procured from Sigma Chemical Co. Samples for GC-MS analysis were prepared by perchloric acid extraction as previously described. [105] The 13C-labeled internal standards were added in two-fold excess of the concentrations of the individual analytes in the tumour tissue to the neutralized PCA extracts. Extracts (5.0 µL) were evaporated under a stream of nitrogen to dryness and were immediately reacted with 5 µL of the sylilating reagent in 15 µL of acetonitrile in 1.5 mL screw capped glass vials and heated to 60°C for 5 minutes. Samples were analyzed on an Agilent 5973 quadrupole GC-MS (Agilent). One microliter of the sample solution was injected onto a 250-µm x 30 m capillary DB-1 (Agilent) column in the splitless injection mode. The mass spectrometer was operated in the electron impact mode (70 eV) and the quadrupole mass analyzer scanned for ions, which corresponded to a loss of 15 mass units (–CH3) from the molecular

ion and the base peak of each analyte and its corresponding 13C-labeled internal standard using selected ion monitoring.

3.3.5. Statistical analysis

Data are expressed as means ± SD with coefficient of variation (COV). Student's *t* test was applied to determine the significance between the groups, with a *P* value of less than .05 considered significant. Grubbs' test was performed using GraphPad to determine whether there were any outliers among the values. The *NF1* PHEOs were not statistically analyzed due to the small sample size.

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4. RESULTS

4.1. Germline mutation prevalence in Hungarian patients with PHEO and/or PGL

Eleven patients were identified to carry mutation in one of the PHEO/PGL associated genes. Together with our previous data demonstrating mutations in *RET* (n=4) and *VHL* (n=4) genes, the prevalence of germline disease-causing mutations in Hungarian patients with apparently sporadic, non-syndromic PHEO/PGL was 21.1% (19/90; 11 of 82 cases, 4 *RET* and 4 *VHL* mutation carriers). For mutation detection bilateral involvement and multiple tumours had the most positive predictive value. The prevalence of bilateral tumours was significantly higher in mutation carriers than in genetically negative cases (8 of 11, 72.8% vs. 3 of 71, 2.1%; $p<0.001$).

The mutation spectrum observed in our patients was heterogeneous, the most frequent mutations were detected in the *SDHB* gene (7 different of which 4 were novel mutations), Three patients had *TMEM127* mutations (two novel) and one had mutation in the *SDHD* gene (Table 9). The chromatograms of all novel mutations identified are presented in Figure 5. All novel *SDHB* mutation have been submitted to TCA Mutation Database and the new *TMEM127* mutations to dbSNP database (http://chromium.lovd.nl /LOVD2/SDH/variants.php?select_db=SDHB&action= view&view=0000838, http://chromium.lovd.nl/LOVD2/SDH/variants.php?select_db=SDHB&action=view& view =0000839; http://chromium.lovd.nl/LOVD2/SDH/variants.php?select_ db=SDHB &action=view&view=0000840; http://chromium.lovd.nl/LOVD2/SDH/ variants.php? select_db=SDHB& action= view&view=0000841).

No mutations in *SDHC, SDHAF2,* and *MAX* were identified in our patients.

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4.1.1. Genotype-phenotype associations

Comparison of the main demographic and clinical data of the genetically positive and negative cases indicated that genetically positive patients were younger, their PHEO/PGL was more frequently malignant, and 72% of cases had bilateral or multiple tumours (Table 8). As expected the malignancy was the highest (3 out of 7 cases) in patients with *SDHB* mutations. Two patients with mutations *SDHB*:c758G>A -Cys253Tyr- and the novel *SDHB*: c.586T>G -Cys196Gly- were lost because of metastatic disease by the age of 35 years. In these patients multiple metastases in bone and liver were observed. In the third case with malignant PGL the novel *SDHB*: c728G>A Cys243Tyr mutation was identified. In this patient an intraabdominal PGL with multiple bone metastases was diagnosed.

Another important finding was that the *SDHB* associated tumours were mainly intraabdominal PGLs (6 out of the 7 cases). In one case with the novel *SDHB* c607G>T Gly203Stop mutation pheochromocytoma and renal cell carcinoma with oncocytic feature was detected at age of 19 years**.** The solid architecture, cytoplasmic inclusions of flocculent material and intratumoural mast cells as the main characteristics for *SDHB* associated renal cell carcinomas could be identified (Figure 6).

Head-neck PGLs were detected in a patient harbouring the *SDHB:* c286+1G/A mutation, and in a patient with *SDHD* c.147-148 insA frameshift mutation. In the latter case an intraabdominal PGL was also removed. After 4-8 years follow-up no malignancy was observed in these cases.

TMEM127 mutations were detected in three patients. Two of them had PHEO (one bilateral) while in the third patient with the novel mutation (*TMEM127*: c467T>A, -Leu155Stop) PHEO and PGL of the head-neck region was also observed. These tumours showed no malignancy. The youngest patient harbouring *TMEM127* associated tumour was 22 years old.

Table 9. Phenotype characteristics of Hungarian patients with PHEO/PGL.

Case	Age	Manifestation	Gene/Mutation
1	33	Paraganglioma (intrabdominal+head/neck, malignant)	*SDHB*:c.758G>A Cys253Tyr
2	32	Paraganglioma (intrabdominal+head/neck, malignant)	*SDHB*: c.586T>G Cys196Gly *
3	31	Paraganglioma (intrabdominal+head/neck)	*SDHB*: c.586T>C Cys196Arg*
4	38	Paraganglioma intraabdominal	*SDHB*: c649C>T Arg217Cys
5	19	Pheochromocytoma + renal cell carcinoma	*SDHB*: c.607G>T Gly203Stop*
6	37	Paraganglioma (head/neck)	*SDHB*: c.286+1G/A,
7	30	Paraganglioma (intraabdominal multiple, malignant)	*SDHB*: c.728G>A Cys243Tyr*
8	32	Paraganglioma (intrabdominal+head/neck)	*SDHD*: c.147-148 insA
9	51	Pheochromocytoma (bilateral) Paraganglioma (intraabdominal and head/neck)	*TMEM127*: c.464T>A Leu155Stop*
10	22	Pheochromocytoma unilateral	*TMEM127*: c419G>A Cys140Tyr
11	47	Pheochromocytoma bilateral	*TMEM127*: c.572delC

*: mutations marked are novel mutations; *SDHB* (ENSG00000117118); *TMEM127 (*ENST00000258439). All novel *SDHB* mutation have been submitted to TCA Mutation Database and the new TMEM127 mutations to dbSNP database. (http://chromium.lovd.nl/LOVD2/SDH/variants.php?select_db=SDHB&action=view&view=0000838, http://chromium.lovd.nl/LOVD2/SDH/variants.php?select_db=SDHB&action=view&view=0000839, http://chromium.lovd.nl/LOVD2/SDH/variants.php?select_db=SDHB&action=view&view=0000840 http://chromium.lovd.nl/LOVD2/SDH/variants.php?select_db=SDHB&action=view&view=0000841)

SDHB – succinate dehydrogenase subunit B, SDHD – succinate dehydrogenase subunit D, TMEM127 – transmembrane protein 127

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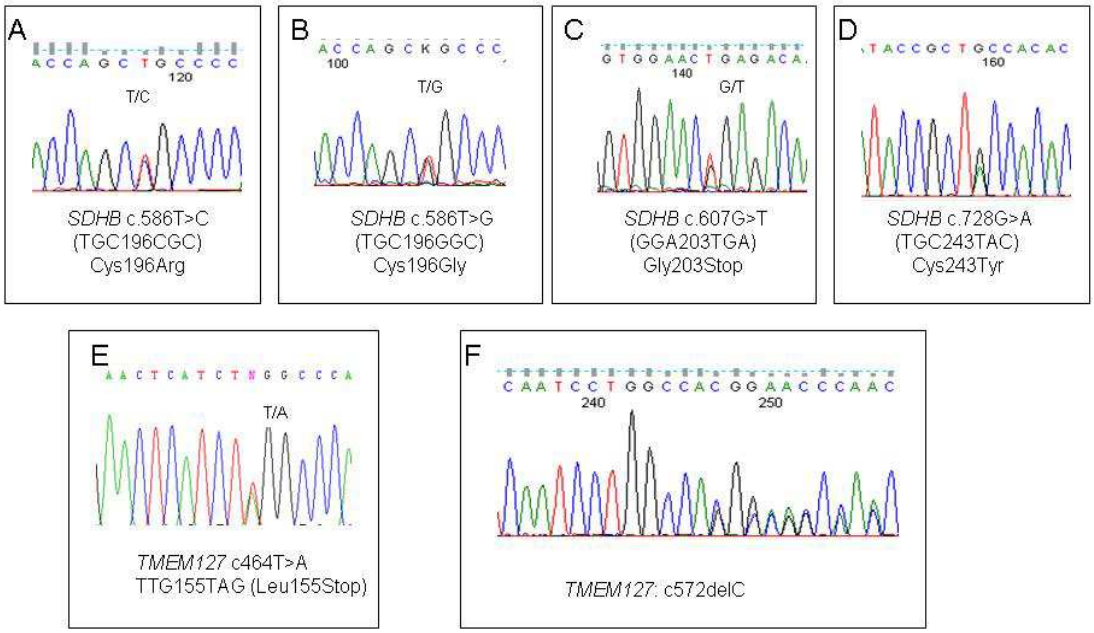
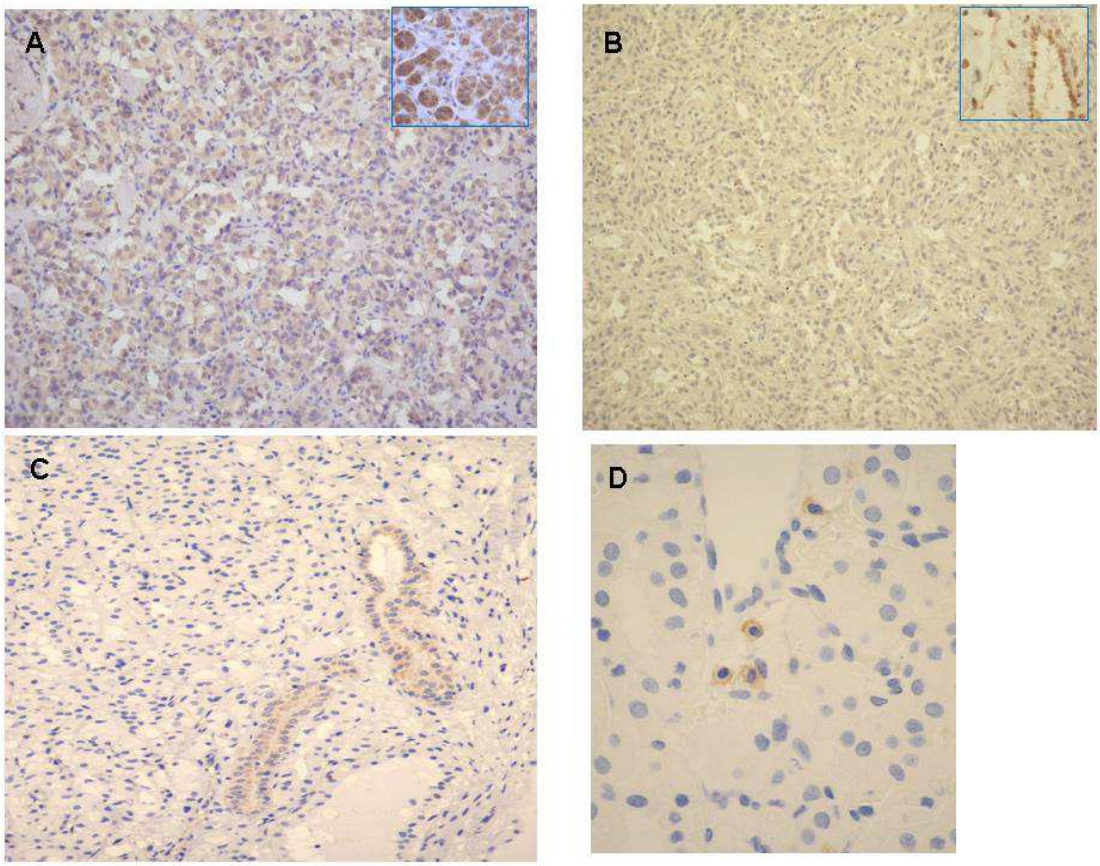
Figure 5. Results of Sanger sequencing and chromatograms of novel germline variants identified in 6 patients with PHEO/PGL.



SDHB - succinate dehydrogenase subunit B, *TMEM127* – transmembrane protein 127, T – thymine, C – cytosine, G – guanine, A – adenine,

Figure 6. Immunohistochemical labeling of tumours associated with novel *SDHB* mutations. Both PGLs and renal cell carcinoma with oncocytic feature associated with *SDHB* mutations showed no SDHB immunohistochemical staining.



Panel A: Intraabdominal PGL associated with *SDHB:* c.586 T > G (Cys196Gly), positive control: adrenocortical cells; **Panel B:** paraganglioma associated with the *SDHB*: c.728G > A (Cys243Tyr) mutation, positive control: endothelial cells; **Panel C:** Renal cell cancer associated with the *SDHB:* c.607G > T (Gly203Stop) mutation. **Panel D:** Entrapped non-neoplastic renal tubules showed positive immunohistochemical labeling for SDHB.

PGL – paraganglioma, SDHB – succinate dehydrogenase subunit B

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4.1.2. The first Hungarian case with extraadrenal pheochromocytoma associated with SDHD gene mutation

Case report

A 33 years-old patient, diagnosed with hypertension 4 years ago (systolic: 120–167 Hgmm, diastolic: 87–110 Hgmm, after 5 minutes of cycling was 251/117 Hgmm), had dizziness, increased perspiration and palpitation. Physical examination showed no abnormalities. The patient had mild hypokalaemia (serum potassium 3,5 mmol/l), mild hypocalcaemia (serum calcium 2,16 mmol/l), all other parameters (glucose level, serum creatinine, cholesterine, triglyceride, liver function, blood count) were normal. The 24 hour fractionated urine metanephrine analysis showed elevated vanillylmandelic-acid (VMA, 18,2 mg/24 h, normal range: 1,8–6,7 mg/24 h) and normetanephrine (2812,9 μg/24 h, normal range: 105–354 μg/24 h), together with normal metanephrine level (101,3 μg/24 h, normal range: 74–297 μg/24 h). Ultrasound, CT and MRI of the adrenal gland showed no abnormalities. The 18-fluoro-DOPA positron emission tomography (18-F-DOPA PET CT) detected a tumour mass resembling for a neuroendocrine tumours at the height of the III. lumbar vertebra before the abdominal aorta. (Figure 7) MRI, later, confirmed the 18-F-DOPA PET identified tumour mass. After preoperative drug administration (α- and β adrenergic receptor blockers) the tumour with a diameter of 3,5cm was laparoscopically resected from the front of the aortic bifurcation. Histological findings confirmed paraganglioma. After tumour resection the symptoms disappeared and the blood pressure normalized. The patient did not present the skin signs typical for neurofibromatosis and MEN2 syndrome was ruled out by normal serum calcitonin level. Family history was unspecific for hereditary syndrome. Regarding the young age he received genetic counselling. After signing informed consent mutation screening was performed for genes causing hereditary syndromes.

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Mutation screening

Genomic DNA was isolated from peripheral blood using commercially available DNA extraction kits (Boehringer Mannheim Corp., Indianapolis, USA) in accordance with the manufacturers' instructions. Mutation analysis of the *VHL, SDHB*, and *SDHD* genes in were performed by bidirectional DNA sequencing of the entire coding region of the *VHL*, *SDHB*, and *SDHD* genes, as previously reported. [99] There were no mutations in the exons and near exons of the *VHL, SDHC* and *SDHB* genes, but there was a heterozygous c. 148-149 insA frameshift mutation found in exon 2 of the *SDHD* gene (Figure 8). The frameshift mutation results in a truncated protein; therefore its pathogeneity is expected.

Family screening

Genetic analysis of family members revealed the presence of the same mutation in his asymptomatic father (c. 148-149 insA frameshift mutation) while the mutation was not present in his mother and brother. Despite detailed physical examination, biochemical (plasma, urine catecholamine) and imaging tests (abdominal CT scan) nor PHEO neither PGL was confirmed in his father.

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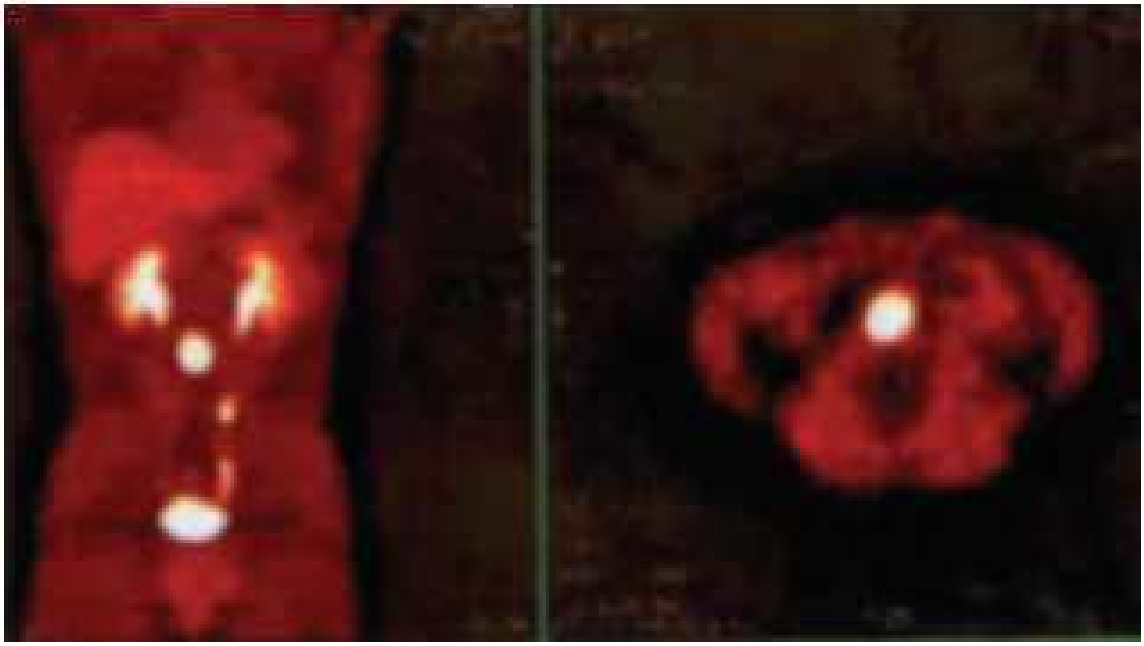
Figure 7. 18-F-DOPA PET/CT scan. Isotope enrichment at the front of the III. lumbar vertebra specific for extra-adrenal pheochromocytoma.



18-F-DOPA PET - 2-^{18}F-fluoro-2-deoxy-D-glucose positron emission tomography

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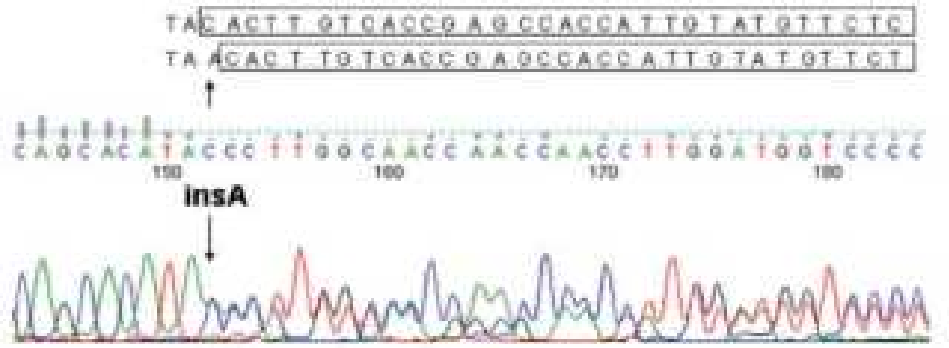
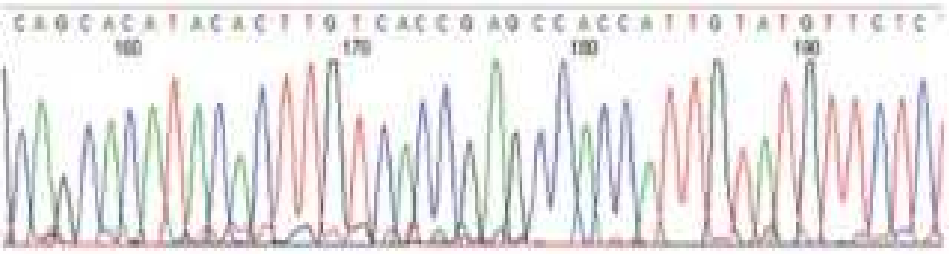
Figure 8. Chromatogram of the c. 148-149 insA frameshift mutation in the *SDHD* gene.



Mutant sequence



Normal (wild) type/sequence

insA – insertion A frameshift mutation

4.2. The G12S polymorphism of the *SDHD* gene as a phenotype modifier in patients with MEN2A syndrome

As mutations of the *SDHx* cause hereditary PHEO/PGL and PHEO is part of MEN2 we hypothesized that variants of *SDHx* genes might be genetic modifiers in MEN2. Therefore all patients with MEN2 from our biobank was tested for *SDHx* mutation. Eight of the 55 patients with MEN2A (15.5%) had the G12S variant, whereas it was absent in the MEN2B and FMTC groups. No patient with sporadic MTC and/or sporadic PHEO carried this variant. Among the 100 population-based, healthy control individuals, only one individual carried this variant (prevalence, 1%) (Table 10). No association between the G12S polymorphism of the S*DHD* gene and the incidence of PHEO or hyperparathyroidism in *RET* mutation carriers was observed, and the age of disease manifestation was similar in G12S carriers and in non-carriers (43±9 versus 40±3 years in probands and 29.6±19.3 versus 32.5±20.5 years in non-carriers). Among probands with *RET* mutations, carriers of the G12S had higher serum calcitonin levels compared with those who did not carry the *SDHD* G12S variant (6,864±11,111 versus 1,250±932 pg/ml), but the difference was not significant. Among family members with *RET* mutations, serum calcitonin levels were similar in G12S carriers and non-carriers 436±876 versus 393±556 pg/ml) (Table 11).

Table 10. Prevalence of the G12S polymorphism of the *SDHD* gene among germline *RET* mutation carriers, patients with sporadic medullary thyroid cancer, patients with sporadic pheochromocytomas, and healthy controls.

MEN2A	FMTC	MEN2B	Sporadic MTC	Sporadic PHEO	Control
8/55 (15.5%)*	0/19 (0%)	0/3 (0%)	0/47 (0%)	0/48 (0%)	1/100 (1%)

*p>0.002 versus control group.

FMTC = familiar medullary thyroid cancer; MEN2A = multiple endocrine neoplasia type 2A; MEN2B = multiple endocrine neoplasia type 2B; MTC = medullary thyroid cancer; Pheo = pheochromocytoma.

Table 11. Clinical presentation, serum calcitonin concentration and the G12S status of patients with MEN2A.

MEN2A (n=55)	**G12S negative**	**G12S positive**
Probands (n=16)	n=13	n=3
Age of presentation (years)	40.1± 9	43±3
Prevalence of		
MTC	12/13	3/3
PHEO	6/13	2/3
PHPT	4/13	1/3
Serum calcitonin; mean ± SD (range)	1206 ± 932 (13-2400)	6864±11111 (124-19690)
Affected family members (n=39)	n=34	n=5
Age of presentation	32.5±19.3	29.6±20.5
Prevalence of		
MTC	22/34	3/5
PHEO	8/34	2/5
PHPT	4/34	1/5
Serum calcitonin mean ± SD(range)	393.8 ± 556 (0-1978)	436.2 ± 876 (0-2000)

SD - standard deviation; MEN2A - multiple endocrine neoplasia type 2A; MTC - medullary thyroid cancer; Pheo - pheochromocytoma; PHPT - primary hyperparathyroidism.

4.3. Biochemical consequences of *SDHx* mutations, succinate to fumarate ratio in *SDHB/D* associated paragangliomas

The present study included 27 tumour samples and *SDHB* silenced and control MPC and MTT cells. The samples included 10 *SDHB* PGLs, 5 *SDHD* PGLs, 2 *NF1* PHEOs, and 10 apparently sporadic PHEOs/PGLs (Table 12).

4.3.1. Succinate concentration in tumour tissues

In *SDHB*-related PGLs, the results showed a mean succinate concentration of 2.7 ± 1.9 mmol/L with the COV of 0.71, in comparison with the apparently sporadic PHEO/PGL group (0.22 ± 0.06 mmol/L; COV of 0.3; $P = .0009$). One *SDHB*-related PGL showed a much higher succinate level than the other *SDHB* tumours (7.9 mmol/L) (Table 13). This sample is a significant outlier ($P < .05$ with a Z value of 2.3). In the *SDHD* group, the mean succinate concentration was 2.1 ± 0.5 mmol/L (COV of 0.24); this value was higher ($P < .05$) than in the apparently sporadic PHEO/PGL group. The *NF1* PHEOs showed a similar mean succinate concentration (0.36 ± 0.16 mmol/L; COV of 0.45) to apparently sporadic PHEOs/PGLs (Table 13).

4.3.2. Fumarate concentration

In *SDHB*-related PGLs, the results showed a mean fumarate concentration of 0.015 ± 0.007 mmol/L with the COV of 0.46, in *SDHD*-related PGLs 0.04 ± 0.03 (COV of 0.62), and in apparently sporadic PHEOs/PGLs 0.038 ± 0.016 mmol/L (COV of 0.43). Fumarate concentrations were significantly lower in the *SDHB*-related compared with *SDHD*-related and apparently sporadic PHEOs/PGLs ($P = .005$, $P = .0008$, respectively). The *NF1* PHEO group showed a mean fumarate concentration of 0.06 ± 0.02 mmol/L (COV of 0.324) (Table 13).

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Table 12. Demographic data of PHEO/PGL samples used in biochemical study

Sample ID	Genetic Background	Localization	Gender
#1	Sporadic	Right adrenal	F
#2	Sporadic	Right adrenal	F
#3	Sporadic	Right adrenal	M
#4	Sporadic	Right adrenal	M
#5	Sporadic	Para-aortic mass	F
#6	Sporadic	Left suprarenal hilum	F
#7	Sporadic	Pleural mass	F
#8	Sporadic	Right adrenal	F
#9	Sporadic	Left adrenal	F
#10	Sporadic	Lumbar spine	F
#11	SDHB	Right ventricular mass	F
#12	SDHB	Para-aortic mass	M
#13	SDHB	Retroperitoneal mass	M
#14	SDHB	Right pericaval PGL	M
#15	SDHB	Retroperitoneal mass	F
#16	SDHB	Subdural/epidural mass	F
#17	SDHB	Thoracic, T1 PGL	M
#18	SDHB	Para-aortic mass	M
#19	SDHB	Paraspinal tumor	M
#20	SDHB	Lung mass	F
#21	NF1	Right adrenal	M
#22	NF1	Right adrenal	M
#23	SDHD	Right carotid body tumor	F
#24	SDHD	Right carotid body tumor	F
#25	SDHD	Right carotid body tumor	F
#26	SDHD	Left carotid body tumor	M
#27	SDHD	Right carotid body tumor	M

SDHB – succinate dehydrogenase subunit B, SDHC - succinate dehydrogenase subunit C, NF1 – neurofibromatosis type 1, F – female, M – male

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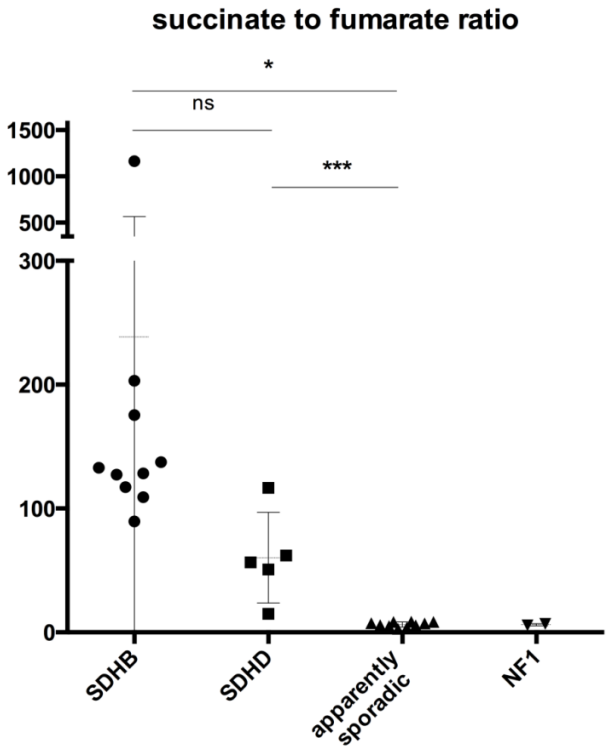
Table 13. Succinate and fumarate concentrations and succinate to fumarate ratio in PHEO/PGL.

Sample ID	Succinate (mmol/L)	Fumarate (mmol/L)	Succinate to fumarate ratio
Sporadic			
#1	0.18	0.02	7.35
#2	0.22	0.03	7.24
#3	0.20	0.02	8.43
#4	0.17	0.06	2.90
#5	0.15	0.04	3.93
#6	0.22	0.04	5.86
#7	0.37	0.07	5.03
#8	0.28	0.03	8.35
#9	0.19	0.03	5.60
#10	0.21	0.02	8.58
Mean	**0.219 ± 0.066**	**0.038±0.016**	**6.3±2.0**
SDHB			
#11	2.79	0.01	203.11
#12	7.92	0.01	1165.31
#13	3.28	0.03	109.16
#14	1.42	0.01	127.34
#15	2.60	0.02	137.48
#16	1.30	0.01	175.47
#17	1.73	0.01	132.95
#18	2.88	0.02	128.30
#19	1.15	0.01	89.50
#20	1.85	0.02	117.30
Mean	**2.692 ± 1.979**	**0.015± 0.007**	**238.6±327.2**
NF1			
#21	0.48	0.07	6.82
#22	0.25	0.04	5.59
SDHD			
#23	1.75	0.03	50.70
#24	2.27	0.02	116.75
#25	2.26	0.04	62.13
#26	1.42	0.09	14.95
#27	2.68	0.05	56.59
Mean	**2.078 ± 0.491**	**0.046 ± 0.029**	**60.24±36.58**

4.3.3. Succinate-to-fumarate ratio in tumour tissues

The mean succinate-to-fumarate ratio was high in *SDHB*- and *SDHD*-related PGLs, 238.6 ± 327.2(COV of 1.37) and 60.24 ± 36.58 (COV of 0.60), in contrast to apparently sporadic PHEOs/PGLs 6.3 ± 2.0 (COV of 0.31) (P = .0376, P = .0003, respectively) (Table 13, Figure 9). The *NF1* PHEOs had a mean succinate-to-fumarate ratio of 6.204 ± 0.87 with the COV of 0.14.

Figure 9. Succinate-to-fumarate ratio in *SDHB, SDHD*, apparently sporadic, and *NF1* tumours.



ns - not significant. * - P< .05; *** - P< .0005.

SDHB - succinate dehydrogenase subunit B, SDHD - succinate dehydrogenase subunit D, NF1 – neurofibromatosis type 1

4.3.4. Succinate-to-fumarate ratio in plasma samples

The plasma mean succinate-to-fumarate ratio showed a slight increase in the *SDHB* and *SDHD* groups (3.15 ± 1.63 and 2.75 ± 1.65) compared with the apparently sporadic group (1.61 ± 0.61), but this difference was not significant.

4.3.5. Succinate-to-fumarate ratio in MPC and MTT cells

The succinate-to-fumarate ratio was significantly higher in *SDHB*-silenced MTT cells compared with control MTT cells; 7.53 vs 2.45 (P = .0115), whereas the small elevation in the *SDHB*-silenced vs control MPC cells was not significant (1.62 vs 1.16, P = .164) (Table 14).

Culture supernatants were analysed in the *SDHB*-silenced and control MPC and MTT cells, but no apparent differences in the succinate-to-fumarate ratio were determined.

The degree of *SDHB* silencing was 62% in MPC and 63% in MTT cells. (Results are not shown.)

Table 14. Mean succinate and fumarate concentrations and succinate to fumarate ratio in *SDHB*-silenced and control MPC and MTT cells (n=5 for each group).

	Succinate (mmol/L)	**Fumarate (mmol/L)**	**Succinate to fumarate ratio**
MPC			
Control	**1.39**	**1.20**	**1.16**
SDHB-silenced	**1.63**	**1.05**	**1.62**
MTT			
Control	**2.25**	**1.26**	**2.45**
SDHB-silenced	**6.86**	**0.85**	**7.53**

MPC – mouse pheochromocytoma, MTT – mouse tumor tissue, SDHB – succinate dehydrogenase subunit B

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5. DISCUSSION

5.1. Germline mutation prevalence in Hungarian patients with pheochromocytoma and/or paraganglioma

PHEO/PGLs are rare catecholamine producing tumours. To date 14 genes have been implicated the genetic susceptibility of PHEO/PGL, which are responsible for the 25–30% of all cases. The American Society of Clinical Oncology have suggested that for patients with a ≥ 10 % chance for carrying a germline mutation genetic testing should be offered [91,106]. Patients with PHEO/PGL are in this group. Currently the genetic analysis of patients with PHEO/PGL includes molecular genetic analysis of *RET, VHL, SDHx, MAX* and *TMEM127* genes. *MAX* and *TMEM127* were identified in 2010, and to date only few studies have been published about the prevalence of mutations of these genes in apparently sporadic cases and only few studies reported genotype-phenotype associations. [45,62107–109] Our current study was initiated to comprehensively analyze the prevalence of germline mutations in our cohort of histologically confirmed non-syndromic patients with PHEO/PGL. Using conventional molecular biological methods we identified 11 germline mutation carriers (*SDHB*=8, *TMEM127*=3), including six novel mutations. These results, together with our previous data on *RET* (n = 4) and *VHL* mutations (n = 4) in Hungarian patients with apparently sporadic, non-syndromic PHEO/PGL shows that 21.1 % of our patients carry mutation in one of the PHEO/PGL susceptibility genes. [99,101] This finding is in line with previously reported data in other populations [45,109] and with a recent review by Brito et al. [108] The mutation spectrum observed in our cohort suggests that no founder mutation is present in the Hungarian population. Genetic studies performed in the past did not include the mutation testing of *KIF1B, EGLN1, TMEM127, MAX* or the recently identified *MDH2* and their prevalence in apparently sporadic PHEO/PGL cases are lacking. Therefore, our findings are also important from this aspect and the results demonstrated that in a population with heterogeneous genetic background the genetic screening should be performed for all of these genes. The novel mutations identified in our cases are considered as disease-causing mutations, because they are either protein truncating mutations

(*TMEM127*. c572delC, *SDHB* Gly203Stop and *TMEM127* Leu155Stop) or they affect residues which are important for protein function and in the same codon other mutations have already been reported as pathogenic (*SDHB* p.Cys196Gly and p.Cys243Tyr) according to the TCA Cycle Gene Mutation Database (http://chromium.liacs.nl). These novel mutations are not listed in any database including dbSNP database (http://www.ncbi.nih.gov/SNP), exome variant server (http://evs.gs. washington.edu/EVS/, version v.0.0.30) and Exac variant (exac.broadinstitute.org/) databases. In addition a negative SDHB immunostaining of tumours associated with *SDHB* p.Cys196Gly, p.Cys243Tyr and Gly203Stop (Fig. 6.) further supports the pathogenic role of *SDHB* mutations in these patients. Genotype-phenotype associations confirmed that the malignant potential is frequently associates with *SDHB* mutations. The presentation and the course of the disease of our case with the *SDHB* Cys196Gly mutation were unique. In this case malignant PGL presenting as a primary PGL in the occipital bone was found. By reviewing the literature only one similar case was found. Kanai et al. presented a 61-year-old male patient diagnosed with multiple paragangliomas including intracranial PGL and osteolytic lesion in the occipital bone. Despite surgical interventions and chemotherapy, the patient died in the fourth year after the diagnosis. No data about the genetic background of this case was reported but the similar behaviour observed in these two cases may raise the pathogenic role of *SDHB*. [110] In addition, a more complex phenotype, including a rare concomitant tumour (PHEO/PGL and renal cell carcinoma) was found in another patient with *SDHB* mutation. Renal cell carcinoma with oncocytic feature has been reported as a hallmark of the *SDHB* associated renal cell carcinomas. [96,111] In our patient the lack of *SDHB* staining confirmed the loss of SDHB protein in tumour tissue while it was kept in renal tubular cells. Based on our and Williamson's results genetic testing of the *SDHB* gene should be offered for patients presenting with renal cell carcinoma with oncocytic features. [96,111]

The lack of mutation of *SDHC* gene is not entirely unexpected among our patients because our patient group consisted of patients having mostly intraabdominal PGLs and PHEOs whereas *SDHC* mutations have been identified exclusively in tumours located at the head

and neck regions. [45,91,107] In addition, sporadic head and neck PGLs may present with less symptoms and maybe they are possibly underdiagnosed. Mutations in *SDHAF2, MAX* and *TMEM127* genes have been reported only in a very few cases. [65,108] In our study no *SDHAF2* and *MAX* mutations were found but *TMEM127* mutations were detected in 3 patients of which 2 mutations proved to be novel. It seems particularly important that *TMEM127* mutations were previously reported only in patients with adrenal PHEOs, but in one of our patient having a novel *TMEM127* mutation bilateral adrenal PHEOs as well as glomus caroticum PGL were detected. This new phenotype, confirmed by later studies indicates that mutations of *TMEM127* can also associate with head and neck PGLs. [62 112,113] In our study the two novel *TMEM127* mutations were truncating mutations strongly suggesting their deleterious nature. The third *TMEM127* mutation was detected in a 22-years-old female patient presenting with unilateral adrenal PHEO. This mutation was already reported by Yao et al. and, surprisingly this seems to be the only *TMEM127* mutation associated with malignant phenotype. [112,113] Toledo et al. reported a six generation family with *TMEM127* mutation and suggested that clinical surveillance in *TMEM127* carriers should be started at the age of 22 years. Our findings indicate that clinical surveillance should be started at earlier age. In our mutation-negative patients only three cases were presented with bilateral or multiple tumours. These patients, together with the 12 patients with malignant phenotype (5 PGL and 7 PHEO) may have mutations of genes which were not investigated in the present study. Testing the *KIF1B, EGLN1, FH, IDH2* and *MDH2* genes by classical methods represents a significant work load and cost; therefore, next generation sequencing based methods would be desired. The clinical follow-up of patients identified with pathogenic, germline mutation and their first-degree relatives is challenging. First of all, in the affected families for the first degree relatives genetic counselling followed by genetic testing should be offered. These tumour syndromes are inherited in an autosomal dominant manner, therefore the chance of inheriting the pathogenic variant is 50%. The *SDHD* gene is maternally imprinted therefore the pathogenic variant is inherited from the paternal side, hence in children inheriting mutation from their mother the development of the disease is extremely unexpected. The penetrance of PHEO/PGL varies significantly between these syndromes. It seems to be very low for

SDHA, SDHB, SDHC, SDHD and *TMEM127* mutations but it is higher for *RET, VHL* and NF1 alterations. Of course the typical manifestations associating with *RET, VHL* and *NF1* mutations are highly penetrant and several times precede the development of PHEO (ie. medullary thyroid cancer in *RET* mutation carriers, renal cell cancer, hemangioblastoma and retina angiomatosis in *VHL* carriers and skin lesions in *NF1* mutation carriers). In these families the routine clinical follow-up includes regular checking for manifestation using laboratory and imaging techniques (summarized by Lenders, [91]).

Investigations show abnormalities in oxygen sensing, HIF1 stabilization [85,114], apoptosis [77]; and increased formation of reactive oxygen species in tumour development associated to *SDHx* mutations.

In the first Hungarian patient with extra-adrenal pheochromocytoma related to *SDHD* gene mutation the heterozygous frameshift c.148-149 insA mutation was detected. This mutation was previously reported in a Turkish family. (www. chromium.liacs.nl/lovd_sdh) [115] The typical transmission for *SDHD* gene mutation and PGL/PHEO syndrome was also seen in our patient, the mutation carrier father was asymptomatic (clinical, biochemical and imaging test showed no abnormalities), therefore he inherited the mutation from his mother. This phenomenon is referred maternal imprinting. [116] In these families the symptoms appear in the children of the asymptomatic or symptomatic mutation carrier males; however the children of the mutation carrier females are usually asymptomatic. The pathogenesis of genomial imprinting is still unclear, which makes it difficult to recognize the inheritance of the disease.

The characteristics of hereditary syndromes with PHEO include manifestation at young age and the increased incidence of these tumours among family members. In addition, in contrast to sporadic cases, hereditary PHEO are mainly bilateral or are located in multiple, extra-adrenal localization compared to non-syndromic, sporadic cases.

In 25-30% of the apparently sporadic pheochromocytoma germline mutations can be identified. In children and young patients mostly mutation of the *RET* and *VHL* genes are affected. [117] However, our case with apparently sporadic extra-adrenal pheochromocytoma with early onset due to mutation in the *SDHD* gene highlighted that even in these younger cases other genes may be mutated. In a family where a mutation carrier was identified

genetic counselling for family members and in mutation carriers clinical testing following the international recommendations are recommended. These recommendations include specific laboratory and imaging testing.

5.2. The G12S polymorphism of the *SDHD* gene as a phenotype modifier in patients with MEN2A syndrome

The phenotypic heterogeneity seen in families with different *RET* mutations, the variation of clinical course within families with the same *RET* mutation, and the results from *RET* transgenic mouse models suggest a potential role of genetic components in phenotype modulation. [56,118]

Polymorphisms of the *RET* gene have been analyzed as such genetic modifiers, but the results from these studies are conflicting. Robledo et al. showed that two *RET* variants (G691S and S904S) may modify the age of onset of MTC in family members [71]; and Tamanaha et al. reported that two intronic polymorphisms of *RET* may modify the phenotype in a large family with G533C *RET* mutation [73], while Baumgartner-Parzer found that the L769L and the IVS14-24 may act as modifiers in some forms of hereditary and sporadic MTC. [119] However, Lesueur et al. were unable to replicate this association in a large cohort of 384 members of MEN2 families from four different European populations. This latter study showed that of the several polymorphisms of *RET*, its co-receptors and ligands, only the synonymous polymorphism (A432A) of the *RET* gene associated weakly with tumour spectra in patients with MEN2A. [74] In MEN2-related MTC *RET* variants have been proposed as genetic susceptibility factors for the development of sporadic MTC: polymorphisms located in coding regions of *RET*; G691S, L769L, S836S, and S904S have been shown to be over-represented in patients with sporadic MTC [120–122] compared with the general population, but others were unable to confirm these associations [123,124] suggesting that variants of RET may be involved in the pathogenesis of sporadic MTC as well.

Germline mutations of *SDHx* genes encoding subunits of the mitochondrial complex II represent a genetic susceptibility for PHEO/PGL. These tumours are derived from cells of

the neural crest, similar to MTC. *RET* mutations also cause PHEO, again suggesting a link between the genetic background of PHEO and MTC. Therefore, it has been assumed that mutations of these genes may be involved in the pathogenesis of MTC. Lima et al. reported a family with C-cell hyperplasia, a pre-cancerous state of MTC, who were proved to have the H50R variant of the *SDHD* gene. [78]

Montani et al. demonstrated an increased frequency of amino acid-coding *SDHx* polymorphisms in patients with sporadic and familial MTC. [76] In addition, a systemic evaluation of genetic variants of the *SDHx* genes among patients with sporadic MTC showed a significant association between the H50R variant and sporadic MTC in Spanish patients, although this observation was absent in an English cohort. [125] Variants of the *SDHx* genes have been implicated in the pathogenesis of various endocrine and non-endocrine tumours, such as Merkel cell carcinoma, carcinoid, papillary thyroid cancer, pituitary tumours and renal cell cancer found in patients with Cowden-like syndrome. [77]

During my PhD thesis work I found that the G12S variant was significantly over-represented among *RET* mutation carriers compared with sporadic MTC, sporadic PHEO, or control individuals. This variant occurred mainly in patients with MEN2A, while Montani et al. detected G12S in a patient with MEN2B harbouring the M918T mutation of the *RET* gene. [76] Interestingly, the prevalence of alterations of the *SDHx* genes in patients with *RET* mutations was similar in our study and the study of Montani et al.. [76] The prevalence of the G12S in the general population is between 2.5% and 5% [126] according to the Leiden Open Variation Database (http://chromium.liacs.nl) [127], which is somewhat higher than in our control population (1%). This difference may be due to differences in the selection criteria applied for controls. Our control group were evaluated for endocrine dysfunction; none of them had signs or symptoms characteristic of thyroid cancer or PHEO. By contrast, population-based controls, frequently anonymous blood donors, have been never tested for these rare conditions. Alternatively, the difference between the studies in prevalence of G12S can also be attributed to the ethnic background of the different populations tested. Our patients and controls were of Hungarian origin, representing an independent entity among Caucasian populations. More importantly, in our study, the high

incidence of the G12S variant among *RET* carriers, especially in those with the MEN2A phenotype, raised the possibility that this variant may have a role in the phenotypic modulation of the disease. However, we were unable to detect significant differences in the clinical presentation between G12S carriers and non-carriers. Whether this failure was a result of the relatively small size of our patient cohort remains to be further investigated. Interestingly, Waldmann et al. reported an increased prevalence of intronic *SDHB* polymorphisms among patients with malignant PHEO compared with patients with benign tumors. [128]

5.3. Biochemical consequences of *SDHx* mutations, succinate to fumarate ratio in *SDHB/D* associated paragangliomas

Using tumour tissue homogenates I found that the tumor tissue succinate-to-fumarate ratio was significantly higher in *SDHB*- and *SDHD*-related PGLs compared to apparently sporadic and *NF1*-related PHEOs/PGLs. Furthermore, *SDHB*-silenced MTT cells showed a similar trend of increased succinate-to-fumarate ratio compared with control MTT cells. These results suggested for the first time that the succinate-to-fumarate ratio can be used as a new metabolic marker for *SDHB/D*-related PHEOs/PGLs.

SDH is the crucial enzyme in energy metabolism that links the tricarboxylic acid cycle, also called the Krebs cycle, to oxidative phosphorylation. [129] In the Krebs cycle, SDH catalyzes the oxidation of succinate to fumarate, whereas as mitochondrial complex II, it transfers electrons to the quinone pool, supporting the reduction of ubiquinone. [129] More than a decade ago, mutations in genes encoding SDH subunits B, C, and D, and more recently mutations in *SDHAF2* and *SDHA*, were discovered to be involved in the pathogenesis of PHEOs/PGLs. [12,15,16,40,41] Mutations in these genes result in impaired function of the SDH enzyme associated with succinate accumulation and loss of fumarate [79]. Succinate accumulation has been shown to result in the inhibition of prolyl hydroxylases and consequently in the impaired degradation of hypoxia-inducible factor α (HIF1-, 2- α). [114] HIF1-, 2- α stabilization affects the activation of many genes promoting tumorigenesis and

cancer development with accelerated aerobic glycolysis (the so-called Warburg effect). [130,131] Reactive oxygen species, which also accumulate due to *SDH* mutations, were found to stabilize HIF-α. [8,132] These and other findings suggest that, indeed, *SDHx*-related PHEOs/PGLs could be viewed as a metabolic disease. [133] Thus, the assessment of metabolic intermediates in these tumours could bring new discoveries, including the introduction of novel biomarkers specifically used in the clinical diagnosis of these unique metabolic tumours. Metabolomics encompasses the characterization of metabolite profiles to genetic or environmental changes in biological samples. [134] There are several different separation and detection methods for analytical procedures of the samples, including nuclear magnetic resonance spectroscopy and GC-MS. Metabolomic analysis is fast and reliable in the identification of metabolite changes in specific tissues, including tumours. [134] Genetic testing and immunohistochemistry are currently excellent methods for the diagnosis of *SDHx* mutations. [86,87] Unfortunately, these methods cannot assess any response of these tumours (eg, to chemo- or radiotherapy, their therapeutic resistance, or follow-up after a therapy is completed). Moreover, these methods cannot detect acute changes in the activity of these tumours; thus, they cannot predict the sudden aggressive behavior and metastatic spread that is often seen in patients with *SDHB* mutations.

By introducing the succinate-to-fumarate ratio as a new marker in these tumours may provide a new opportunity to not only diagnose but also monitor their behaviour and therapeutic responses. Currently such monitoring would require a tumour sample to be obtained; we predict that in the near future plasma samples could also be used to assess these tumours as described above. This will be based on large prospective studies, as well as the introduction of more sensitive GC-MS methods. Because the pathogenesis of these tumours is primarily based on mitochondrial damage tightly linked to the Krebs cycle and the Warburg effect, we predict that other important metabolites will soon be introduced and used in clinical assessment with the succinate-to-fumarate ratio.

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6. CONCLUSION

I summarized clinical, demographic and genetic data of Hungarian patients with apparently sporadic PHEO/PGL. Using a comprehensive mutational screening of a large series of patients with PHEO/PGL, I determined the prevalence of disease-causing mutations in this patient group. The most frequent mutations were detected in the *SDHB, TMEM127, RET and VHL* gene. This heterogeneous genetic background with six novel mutations observed in Hungarian patients was similar to other populations where no founder mutations are present. The genetic screening offered for PHEO/PGL patients in this population should cover all of the genes identified to date but the first gene for testing should be the *SDHB* for patients with intraabdominal PGL especially with malignant phenotype. The novel genotype-phenotype associations revealed may contribute to improvement of diagnostic approaches and may help to achieve a better clinical follow up of patients with PHEO/PGL. Both laboratory workload and cost of testing of all genes are still significant, but phenotype oriented guidelines allow us to set up an order of genes tested, after a negative result the remaining genes should be also examined. For most effective work the optimum would be to exclude some of the syndrome-associated genes based on the obvious phenotype features (i.e. because of typical manifestation the NF1 gene is rarely tested) and all remaining genes would be tested at the same time. Testing *KIF1B, EGLN1, FH, IDH2* and *MDH2* genes by next generation sequencing based methods would also be desired. The clinical follow-up of patients identified with pathogenic, germline mutations and their first-degree relatives is challenging. First of all, in the affected families for all first degree relatives genetic counselling followed by genetic testing should be offered.

Beside the disease-causing *SDHx* mutation I found a significantly higher prevalence of the G12S variant of the *SDHD* gene among germline *RET* mutation carriers presenting with MEN2A compared to the control group. The high prevalence of the G12S variant in these patients supports its genetic modifier role, however, we were unable to detect significant differences in the clinical presentation between G12S carriers and non-carriers. This proposal remains to be established.

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For the first time I was able to demonstrate that the succinate-to-fumarate ratio could be used as a new metabolic marker for the presence of *SDHB/D*-related PGLs. Accumulation of succinate result in the inhibition of prolyl hydroxylases and consequently in the impaired degradation of hypoxia-inducible factor α (HIF1-, 2- α). [114] HIF1-, 2-α stabilization has an impact on genes promoting tumorigenesis and cancer development with accelerated aerobic glycolysis. [130,131] Based on the literature and my results, through a large prospective clinical study including other *SDH* PHEOs/PGLs, it would be possible to determine the diagnostic accuracy of succinate-to-fumarate ratio in the diagnosis of PHEO/PGL. Furthermore, following the confirmation of our initial results, we may hypothesize that intratumoural and perhaps plasma changes in the succinate-to-fumarate ratio will serve as an important indicator of potential therapies directed toward mutated SDH proteins. [135]

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7. SUMMARY

Succinate dehydrogenase links the citric acid cycle and the oxidative phosphorylation. It consists of four subunits (SDHA, SDHB, SDHC, SDHD), which are encoded in the nuclear genome. Mutations can occur in all subunit genes causing familial paraganglioma syndromes.

Pheochromocytomas and paragangliomas are tumours deriving from the chromaffin cells of the adrenal gland and the sympathetic and parasympathetic ganglions, respectively. The symptoms are due to the extreme catecholamine secretion and/or the pressure of the surrounding tissue. In 25-30% of the apparently sporadic cases germline mutations of the *RET, VHL, NF1, SDHx, SDHAF2, TMEM127* and *MAX* genes are identified.

In the mutation screening analysis of the Hungarian population mutations in the *RET, VHL, SDHx, TMEM127, MAX* genes were identified, which showed the same distribution as described in literature. Six novel, possible disease causing mutations were identified in SDHB and *TMEM127* genes and it has been confirmed that *SDHB*-related tumours have a high risk of malignancy and are mostly associated with abdominal paragangliomas. The prevalence of the G12S variant of *SDHD* gene is high in multiple endocrine neoplasia type 2A patients harbouring *RET* mutation. The presence of G12S variant seems to play a role as phenotype modifier in MEN2 patients, which needs to be clarified. Due to mutations in the *SDHx* genes the enzyme function is disturbed and succinate can accumulate. In *SDHB/D*-related paragangliomas the succinate-to-fumarate ratio was significantly higher compared to NF1 tumours and controls. This is the first time to present that succinate-to-fumarate ratio can be a new marker in the diagnosis of *SDHB/D*-related paragangliomas. This current investigation hypothesizes that plasma succinate-to-fumarate ratio could be a marker for tumour follow up and treatment in the future.

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7. ÖSSZEFOGLALÁS

A citrát ciklust és az oxidatív foszforilációt egy négy alegységből álló enzim, a szukcinát dehidrogenáz köti össze. Mind a négy alegységet (SDHA, SDHB, SDHC, SDHD) kódoló gén a nukleáris genomban kódolt és az esetlegesen előforduló mutáció esetén hibás enzim jön létre, mely familiáris paraganglióma szindrómák kialakulásához vezethet.

A phaeochromocytómák és paragangliómák (PHEO/PGL) a mellékvese velőállományának kromaffin sejtjeiből illetve ritkábban a szimpatikus vagy paraszimpatikus ganglionsejtekből kiinduló daganatok. A klinikai tüneteket a daganatban képződő katecholaminok okozzák, de bizonyos esetekben nyomási tüneteket is jelentkezhetnek. Általában sporadikusan fordulnak elő, de 25-30%-ukban ki lehet mutatni a *RET, VHL, NF1, SDHx, SDHAF2, TMEM127*és *MAX* gének csírasejtes mutációit.

A hazai sporadikus PHEO/PGL populációban végzett mutáció analízis segítségével bebizonyosodott, hogy a *RET, VHL, SDHx, TMEM127, MAX* génmutációk előfordulása megegyezik az irodalomban közölt adatokkal. Hat új, feltehetően betegség okozó mutáció került bemutatásra az *SDHB* és *TMEM127* génekben, valamint igazolódott, sporadikus intraabdominális PGL betegekben az *SDHB* mutáció a leggyakoribb, és az *SDHB*-hez társult betegségek malignusak. Az *SDHD* gén G12S variánsa nagyobb gyakorisággal fordult elő a *RET* mutációt hordozó multiplex endokrin neoplázia 2A szindrómában szenvedő betegekben. A G12S variáns előfordulása ezen betegekben feltételezi genetikus módosító szerepét, mely jelenleg még tisztázásra vár. Az *SDHx* gén mutációk következtében sérült funkciójú enzim jön létre, ami a szukcinát szintjének emelkedésével jár. Az *SDHB/D*-hez társult PGL szövetmintáiban a szukcinát-fumarát aránya szignifikánsan magasabb volt a sporadikus és *NF1* PGL-hoz képest. Első alkalommal sikerült bemutatni, hogy a szukcinát-fumarát arány, mint lehetséges új metabolikus marker alkalmazható lenne a *SDHB/D* génmutációhoz társult PGL jelenlétének kimutatásában. A jelenlegi vizsgálat feltételezi, hogy a későbbiekben a plazma szukcinát-fumarát arány a daganat nyomonkövetésében, esetlegesen a kezelésében nyújthat segítséget.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

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9.2. Publications not related to the theme of the PhD thesis

Fliedner SM, Kaludercic N, Jiang XS, Hansikova H, Hajkova Z, Sladkova J, Limpuangthip A, Backlund PS, Wesley R, Martiniova L, Jochmanova I, **Lendvai NK**, Breza J, Yergey AL, Paolocci N, Tischler AS, Zeman J, Porter FD, Lehnert H, Pacak K. (2012). Warburg effect's manifestation in aggressive pheochromocytomas and paragangliomas: insights from a mouse cell model applied to human tumor tissue. *PLoS One.* 7:e40949.

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