# THE PROFILE OF GENETIC MUTATIONS IN PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS IN SOUTH AFRICAN PATIENTS

by

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Thesis submitted in fulfilment of the requirements for the degree

**MMed (Anatomical Pathology)**

in the

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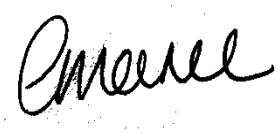
**April 2020**

**DECLARATION**

I, Liezanne Maree, declare that the coursework Master's Degree mini-dissertation that I herewith submit in a publishable manuscript format for the Master's Degree qualification in Anatomical Pathology at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.



**April 2020**

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**Liezanne Maree** **Date**

**ACKNOWLEDGEMENTS**

I would like to thank the following:

First, I would like to express my sincere gratitude to my supervisor, Prof J. Goedhals, for her motivation and expert guidance throughout the entire research process.

I would also like to extend my gratitude to Mr A. Bester and Me. S. Pretorius for their continuous support and enthusiasm, and their generous time and effort that they have invested in my research.

**FINANCIAL SUPPORT**

This research was funded by the Department of Surgery Research Fund, University of the Free State.

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**ABSTRACT**

**Background:** Pheochromocytomas (PCCs) are rare neoplasms of the adrenal glands. Extra-adrenal pheochromocytomas, or paragangliomas (PGLs), most commonly involve the carotid body and middle ear. Both germline and somatic variants are associated with their pathogenesis, and identification of a specific genetic variant guides clinical management in patients and their families. The genetic heterogeneity of PCCs/PGLs is distinct, creating the possibility of personalized, genomics-driven therapy. Next-generation sequencing allows massive parallel sequencing of all genes of interest in a single, cost-effective run. Internationally, extensive research has been done regarding the genetic make-up of PCCs/ PGLs, and the genes most commonly involved include *SDHB, SDHD, RET,* and *VHL*.

**Aim:** To describe the genetic variants in PCCs/PGLs in the South African population.

**Methods:** A retrospective study was performed. Ninety eight of the most recent cases with sufficient tissue available in wax blocks were included. Manual DNA extraction and subsequent DNA sequencing was performed, and 16 genes of interest were assessed for possible variants. These included *EGLN1*, *EPAS1*, *FH*, *HRAS*, *IDH1*, *KIF1B*, *MAX*, *NF1*, *RET*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *TMEM127* and *VHL*.

**Results**: Thirty one of the 98 cases were sequenced. In the remaining cases the DNA quality was inadequate. Ninety three variants were detected with 32 synonymous variants, 54 missense variants, and 7 truncating variants. Nine pathogenic or likely pathogenic variants were identified in total, involving the *NF1*, *KIF1B*, *RET, SDHB* and *TMEM127* genes. Most variants identified were predicted benign, likely benign and benign variants or variants of unknown significance.

**Conclusion:** The profile of pathogenic or likely pathogenic variants identified in this study differs somewhat from that described in the literature with *NF1* and *KIF1B* most commonly involved. In addition, no pathogenic *SDHD* or *VHL* variants were found in this study. However, the number of patient's is small and additional data are required to determine the true genetic profile in South African patients.

**Keywords:** Pheochromocytoma, paraganglioma, next-generation sequencing, genetic variants, germline, sporadic, familial, syndromic.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| PCC/s | - | Pheochromocytoma/s |
| PGL/s | - | Paraganglioma/s |
| SDH | - | Succinate dehydrogenase complex |
| SDHA | - | Succinate dehydrogenase complex Subunit A |
| SDHB | - | Succinate dehydrogenase complex Subunit B |
| SDHC | - | Succinate dehydrogenase complex Subunit C |
| SDHD | - | Succinate dehydrogenase complex Subunit D |
| SDHDF2 | - | Succinate dehydrogenase complex Subunit DF2 |
| WHO | - | World Health Organization |
| MEN2A | - | Multiple endocrine neoplasia Type 2A |
| MEN2B | - | Multiple endocrine neoplasia Type 2B |
| NF1 | - | Neurofibromatosis Type 1 |
| VHL | - | Von Hippel-Lindau |
| MEN1 | - | Multiple endocrine neoplasia Type 1 |
| DNA | - | Deoxyribonucleic acid |
| HIFs | - | Hypoxia-Inducible Factors |

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# CHAPTER 1

# LITERATURE REVIEW

## Background

Pheochromocytomas (PCCs) are rare neoplasms of the adrenal chromaffin cells (1). Chromaffin cells are neuroendocrine cells found mostly in the medulla of the adrenal glands, which synthesise and secrete catecholamines (adrenaline and noradrenaline) under the control of the sympathetic nervous system (2). During embryological development, the chromaffin cells migrate from the neural crest to the adrenal medulla, but also to areas adjacent to the sympathetic ganglia and parasympathetic paraganglia (3). The sympathetic ganglia are located in the trunk along the prevertebral and paravertebral chains in the thorax, abdomen and pelvis (4). The parasympathetic paraganglia are usually located in the vicinity of major arteries and nerves, most commonly in the head and neck and along the branches of the carotid arteries and vagus nerve (4). The largest extra-adrenal cluster of chromaffin cells is known as the organ of Zuckerkandl (3). In lower concentrations, extra-adrenal chromaffin cells also reside in the bladder wall, prostate, reproductive organs and behind the liver (3). Neoplasms originating from chromaffin cells outside the adrenal gland, which historically have been called extra-adrenal pheochromocytomas, are generally referred to as paragangliomas (PGLs), with the two most common extra-adrenal sites being the carotid body and the jugulo-tympanic apparatus of the middle ear (5).

The mean age at diagnosis of pheochromocytomas and paragangliomas is the fourth to fifth decades, although familial cases tend to occur in a slightly younger age group, and about 10-20% of cases affect the paediatric population (6). Males and females are roughly equally affected (7), although head-and-neck paragangliomas are more frequently associated with a female predominance, which is even more pronounced at higher altitudes, in which case the female-to-male ratio is 8:1 (8). Patients usually present with a characteristic triad of sweating attacks, tachycardia and headaches related to catecholamine hypersecretion (9). Other symptoms include anxiety, tremors, nausea, pallor and abdominal or chest pain. In rare instances, the tumours can cause severe cardiovascular or neurological manifestations such as shock, heart failure, seizures and stroke, all of which can be life-threatening (2). Generally, tumours arising from the sympathetic nervous system secrete catecholamines and are therefore functional, whereas tumours that arise from the parasympathetic nervous system which mostly do not secrete catecholamines are endocrinologically silent (3,5,7,9). These non-

functioning tumours usually present as slow-growing space-occupying masses with symptoms such as pain, hearing disturbances, hoarseness and dysphagia depending on their location (2). Patients with functional tumours also commonly suffer from severe hypertension of abrupt onset that in some cases is unresponsive to treatment (2,7,10). Cardiovascular complications of PCCs and PGLs include hypertrophy of the left ventricle, dilated cardiomyopathy and catecholamine-induced myocarditis which may result in cardiac failure and even death (11). The diagnosis is based on increased urinary excretion of catecholamines or its metabolites, supplemented with pre-operative imaging to localise the primary tumour (7).

Since the year 2000, the traditionally used 'rule-of-tens' (10% familial, 10% malignant, 10% extra-adrenal) no longer holds (12). The current conclusion is that up to 40% of PCCs and PGLs are inherited (1), about 15% occur in extra-adrenal locations and the frequency of malignancy ranges from 3-36% (2,7), where metastases can occur up to twenty years after removal of the primary tumour, most commonly to local lymph nodes, bone, liver and lung (2,11).

The incidence of these tumours ranges from 1 per 2500-6500 people (13), but this is possibly a huge underestimate. This non-specific value is probably due to the fact that these tumours are quite often missed, and in many instances only diagnosed post-mortem (13). This may be due to a low index of suspicion on the physicians' side, the non-specific nature of the symptoms, as well as the fact that approximately 30% of these tumours are non-functional, as previously mentioned (2).

Even though these tumours are quite rare and the great majority of PCCs and PGLs are benign, prompt clinical detection is of the utmost importance since they are potentially lethal. This is not only due to their ability to secrete catecholamines but also their potentially malignant behaviour, defined by the presence of distant metastasis (11). Currently, the likelihood of malignancy cannot be clinically predicted with certainty, however, certain gene expression profiles, like succinate dehydrogenase complex subunit B (*SDHB*) mutations, and histological features, like high proliferative activity, increased cellularity, invasive growth, and comedo necrosis, might be suggestive of a possible risk of malignant behaviour (7). No single risk stratification scheme is currently endorsed or in widespread use, and the current understanding is reflected in the 2017 WHO classification, which is that all PCCs and PGLs should be regarded as having metastatic potential (7). The overall prognosis for malignant disease is poor with a 5-year mortality rate of more than 50% (2,10,14).

PCCs and PGLs occur in all races but have mainly been described in Caucasians. Research relating to PCCs and PGLs in African patients is limited and consists mainly of single cases and small series (11), that reported a similar incidence, age and sex distribution, symptomatology, macroscopic appearance, histology and complications, to that seen in Western countries (15). More recently, the similarities between Caucasian and African patients have been confirmed in a 30-year South-African audit done between 1980 and 2009 which included 54 black patients (11). The lack of data from the African continent as a whole is probably due to limited health resources in general, as well as limited laboratory and radiological facilities to aid in the diagnosis of PCCs and PGLs (11).

Histologically, PCCs and PGLs usually show a classic architectural pattern with so-called 'Zellballen' consisting of nests of cells with surrounding sustentacular cells and delicate capillaries (7). Trabecular or solid growth patterns can also be observed (7). Overall, PCCs and PGLs are quite vascular neoplasms explained by the activation of the hypoxia-inducible factor pathway (16). The 'zellballen' are positive for synaptophysin and chromogranin while the sustentacular cells can be highlighted with an S100 immunohistochemical stain (Figure 1) (7).

PCCs and PGLs can be classified as syndromic, familial or sporadic and both germline and somatic variants are associated with the development of PCCs and PGLs (14). A germline variant is inherited through the germ cells and is therefore present in every cell in the body and will be passed on to future generations. Most germline variants associated with PCCs and PGLs are inherited in an autosomal dominant fashion (7,11), keeping in mind that a small percentage have a parent-of-origin effect and therefore tumours will only develop in these individuals if the variant was inherited through the paternal line (5). In contrast, a somatic variant is acquired after fertilisation, is only present in the tumour cells and is not inherited by any offspring. Up to 40% of PCCs and PGLS are associated with germline variants (1,5,9,14,16) and are then usually seen in the context of a specific familial syndrome (5,9,14,16). PCCs and PGLs occurring in a familial setting usually present at a much younger age than sporadic tumours, and are more often bilateral and/or multifocal (17). However, the majority of tumours will present as isolated sporadic cases that remain genetically unexplained (18).

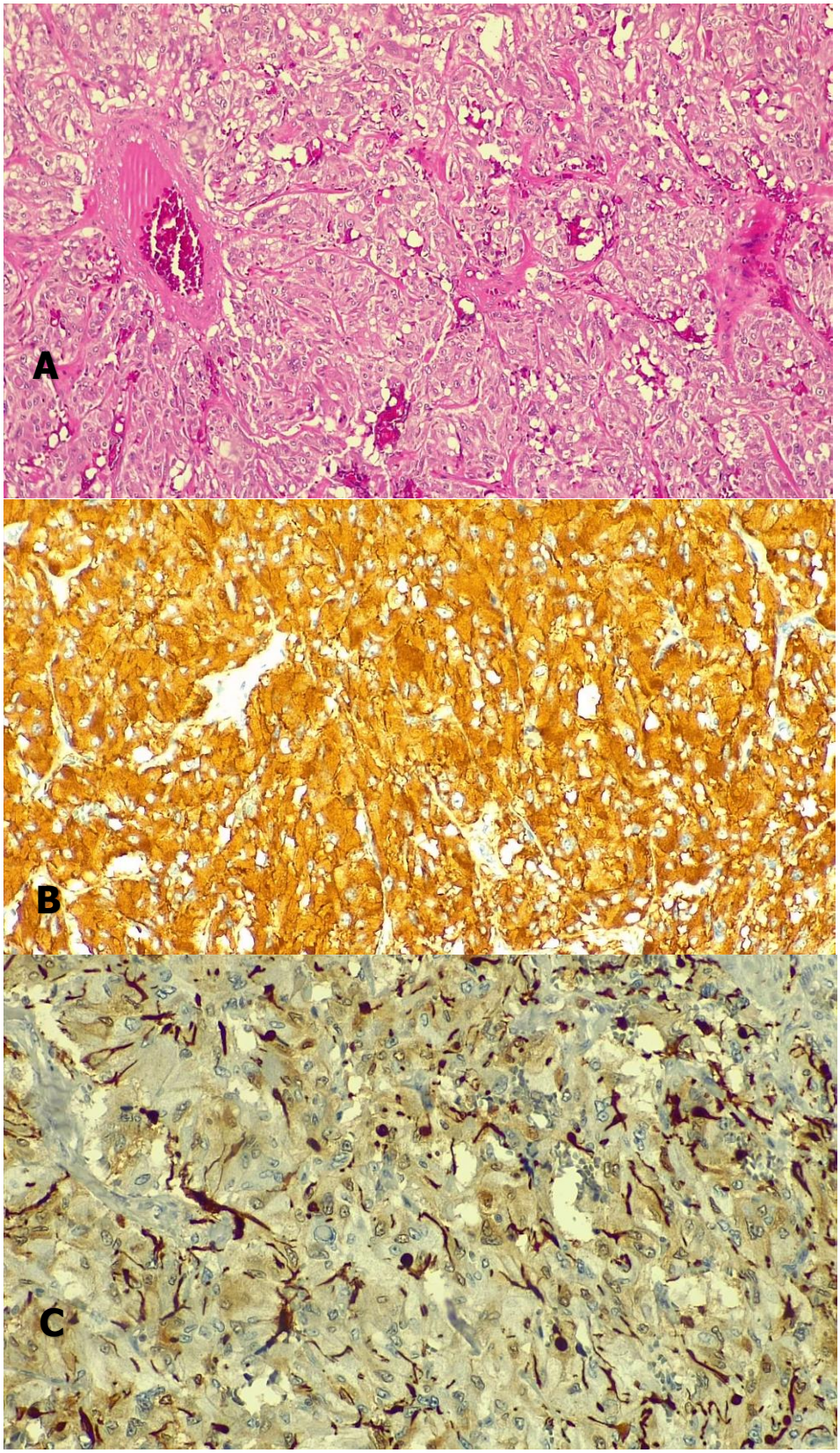


**Figure 1. (a) H&E of a pheochromocytoma with 'zellballen'; (b) Synaptophysin positivity; (c) S100 positivity highlighting the sustentacular cells.**

The familial syndromes, with their associated germline-variants known to be involved in the tumorigenesis of PCCs and PGLs, are summarised in Table 1 (5,9,13,16,19).

**Table 1. Familial syndromes with the affected gene, malignant potential, incidence of PCCs and PGLs and other clinical associations.**

| Syndrome | Gene | Malignancy | Incidence of PCCs / PGLs | Other clinical associations |
|---|---|---|---|---|
| Multiple endocrine neoplasia type 2A (MEN2A) | *RET* (10q11.2) | Rare | 50 % | - Medullary thyroid carcinoma<br>- Hyperparathyroidism |
| Multiple endocrine neoplasia type 2B (MEN2B) | *RET* (10q11.2) | Rare | 50 % | - Medullary thyroid carcinoma<br>- Marfanoid habitus<br>- Mucosal ganglioneuromas |
| Neurofibromatosis type 1 (NF1) | *NF1* (17q11.2) | ± 12% | Up to 6 % | - Cafè au lait spots<br>- Neurofibromas<br>- Axillary freckling<br>- Benign iris hamartomas (Lisch nodules)<br>- Optic nerve gliomas |
| Von Hippel-Lindau (VHL) | *VHL* (3p25) | < 5% | Up to 20 % | - CNS haemangioblastomas<br>- Renal cell carcinoma<br>- Pancreas neuroendocrine tumours<br>- Retinal angiomas |
| Familial Paraganglioma 1 | *SDHD* (11q23) | < 5% | Up to 75 %; with parent of origin (paternal) effect | - Renal cell carcinoma<br>- Gastrointestinal stromal tumours<br>- Pituitary adenomas |
| Familial Paraganglioma 2 | *SDHDF2* (11q12) | Unknown | Unclear with a parent of origin effect | |
| Familial Paraganglioma 3 | *SDHC* (1q23) | Rare | Rare | - Renal cell carcinoma<br>- Gastrointestinal stromal tumours<br>- Pituitary adenomas |
| Familial Paraganglioma 4 | *SDHB* (1p36) | 31%-71% | Up to 50 % | - Renal cell carcinoma<br>- Gastrointestinal stromal tumours<br>- Pituitary adenomas |
| Familial Paraganglioma 5 | *SDHA* (5p15) | 0% - 14% | Unclear | - Renal cell carcinoma<br>- Gastrointestinal stromal tumours<br>- Pituitary adenomas |

A small fraction of PCCs and PGLs are rarely associated with other syndromes, including Carney's triad, Carney-Stratakis syndrome and very rarely, MEN1 (2).

**Importance of identifying variant-positive individuals:**

Identification of a specific gene variant is of the utmost importance because it can guide clinical management in variant-positive patients and their families in terms of the following aspects (1,4,9,16,20,21):

- Accurate diagnosis:
  - Patients should be investigated for the presence of other supra- and infra-diaphragmatic disease, with or without catecholamine secretion, allowing accurate risk stratification,
  - Patients should also be investigated for the presence of other associated tumours in a patient with a syndromic disorder, with the possibility of prophylactic therapy.
- Prognosis:
  - Patients with a germline variant, especially *SDHB*-mutant tumours, have an overall poor prognosis and require close and optimal follow-up to monitor for any recurrences or possible malignant transformation.
- Family screening:
  - Diagnosis of a hereditary disease also allows genetic screening in first-degree relatives of the index subject, thus clarifying the genetic status of asymptomatic family members and possibly even enabling treatment in earlier stage disease when intervention may be more effective,
  - Even establishing a somatic variant, versus a germline variant avoids further genetic screening of relatives, and thus reducing considerable anxiety.
- Therapeutic opportunities:
  - Identification of specific genetic variants can in the near future, guide targeted molecular-based therapy, especially if surgery alone is not curative.

All of this will then lead to:

- overall earlier diagnosis,
- earlier-stage treatment,
- regular surveillance,
- better prognosis and,
- overall improved care for patients and their relatives.

**Who to test:**

PCCs and PGLs are considered to be the tumours with the highest frequency of a hereditary basis (22) and given this high frequency of germline variants, current practices recommend that genetic testing is at least considered in each patient diagnosed with a PCC or PGL, in concordance with international guidelines (10,22). However, that does not necessarily imply that genetic testing *should* be performed in *all* patients. Genetic testing is considered to have limited value in a patient with no family history, no syndromic or malignant features, and with only unilateral disease, specifically in patients that only present above 45 years of age (21). On the other side of the spectrum, young age at presentation, positive family history and multifocal disease are recognizable reasons for prioritizing patients for genetic testing (1,17). Overall, the importance of diagnosing an inheritable disease in an at-risk family must be weighed against the substantial financial costs of genetic testing (1), and should just be considered when the results will affect future medical management for the patient and relatives.

Criteria for identifying patients that would benefit from genetic screening include the following (3):

- Patients with clinical features or a family history possibly consistent with an inherited syndrome known to be associated with PCCs and PGLs,
- Patients with a positive family history, up to and including a third-generation pedigree, of PCCs and PGLs, keeping in mind the parent-of-origin effect seen in *SDHD*, *SDHAF2* and *MAX* genes,
- Patients with bilateral and/or multifocal disease,
- Patients with metastatic disease indicative of malignancy, which in itself is a strong indicator of a germline variant,
- Patients with extra-adrenal disease,
- Patients with early age-onset disease, specifically less than 45 years of age.

Despite all the positive rationales for genetic testing in the correct setting, past research has identified significant barriers to genetic testing (23). In general, cultural beliefs and attitudes as well as fear of possible discrimination, concerns about privacy and confidentiality, a lack of knowledge and limited insight on the patient's side, together with a concern about what to do with any significant results, have all been identified as contributable barriers to prompt and actionable genetic testing (23).

**The diverse mutational landscape of PCCs and PGLs:**

For the past two decades, the genetic landscape of PCCs and PGLs has been ever-changing. Before 2000 it was thought that inherited PCCs and PGLs were only attributed to MEN2, NF1 and VHL (3,7,9). In 2000, germline variants in the *SDHB* and *SDHD* genes, associated with paraganglioma-pheochromocytoma syndromes, were identified (9,12). By 2006, the European Network for the Study of Adrenal Tumours Pheochromocytoma Working Group's recommendations for the genetic workup of affected patients only included testing for *VHL* and *SDHB* variants, with testing for *SDHD* variants if no variants were detected in the first two genes (12). Clinicians and pathologists should also keep in mind, that patients who appear to have an inherited disorder but do not have a variant of any currently known genes, should preferably have DNA banked to allow for any future molecular analysis (3). However, it should be remembered that even though comprehensive genetic analysis does increase the likelihood of identifying an inherited case, it will unfortunately also identify rare genetic variants that might be problematic when trying to classify them as benign polymorphisms or actual pathogenic variants (3).

A very distinct feature of PCCs and PGLs is their genetic heterogeneity which is also directly linked to the varied downstream activated pathways and to date multiple genes have been confirmed to participate in the pathogenesis of PCCs and PGLs, with new ones added annually (1,3,10,13,17,19,21,24–26).

Three classes of major susceptibility genes, each with distinct molecular profiles have been described (2,5,14,17,18,21,22,27):

- Cluster 1 Genes:

Cluster 1 variants are associated with the pseudo-hypoxic signature and overexpression of angiogenesis factors, and mainly correspond to SDH- and VHL-related tumours.

- Cluster1A Genes: ***SDHA, SDHB, SDHC, SDHD, SDHAF2, MDH2, FH***
- Cluster1B Genes: ***VHL, EPAS1 (HIF2A), ELGN1 (PHD2), IDH1***

Hypoxia-Inducible Factors (HIFs) are transcription factors which are activated under hypoxic or pseudo-hypoxic conditions. Under normoxic conditions, these transcription factors are degraded under the regulation of the VHL protein, after hydroxylation performed by members

of the EGLN/PHD family. In the absence of a functional VHL protein, HIFs accumulate and induce the transcriptions of genes involved in angiogenesis. Therefore, a deficient amount of functional VHL protein causes the same response as hypoxia, referred to as pseudo-hypoxia.

The SDH enzyme complex is also associated with a pseudo-hypoxic response. Inactivating variants of *SDH* causes downstream accumulation of succinate which causes inhibition of EGLN activity, leading to HIFs stabilization and activation.

- Cluster 2:

Cluster 2 variants, predominantly *RET* and *NF1* variants, are associated with increased activation of kinase signalling pathways RAS/RAF/MAPK and PI3K-AKT-mTOR :

  - ***RET, NF1, KIF1Bß, MAX, TMEM127***

Oncogenic activation of RET triggers activation of both kinase cascades, with subsequent cell proliferation and uncontrolled growth promotion.

The NF1 protein, neurofibromin, promotes the conversion of RAS into its inactive form, and therefore *NF1* variants also lead to the activation of the RAS/RAF/MAPK pathway. *NF1* variants can also activate the PI3-AKT pathway, which is dependant on increased RAS activity.

- Other genes are also implicated in the development of PCCs and PGLs, but their exact role in pathogenesis is yet to be established:
  - ***GDNF, HRAS, KRAS, GNAS, CDKN2A, TP53, BAP1, BRCA1&2, ATRX, KMT2D, HIF2, SLC25A11, GOT2, DNMT3A, BRAF, TERT, MET, CDH1, KMT2D, FHIT, MEN1***

**Future genomic-driven therapy:**

- The substantial genetic heterogeneity of PCCs and PGLs, with the subsequent heterogeneity in the downstream activated molecular pathways, creates the possibility of future personalized, genomics-driven therapy based on the underlying mutation, as well as the determination and implementation of diagnostic- and prognostic biomarkers of malignancy useful in overall patient care (10,14). This molecular-based therapy would comprise of antiangiogenic drugs for Cluster 1 tumours and mTOR or RAS-RAF pathway inhibitors for tumours belonging to

Cluster 2 (14,21). Even drugs targeting epigenetic pathways could be a therapeutic option, specifically for *FH*-related and *SDH*-related malignant PCCs and PGLs (21).

**Why 'Next-Generation-Sequencing'?:**

The genes most commonly mutated according to international literature are *SDHB, SDHD, RET* and *VHL*, and some of the earlier literature recommended genetic testing for these specific four genes when a patient presented with a PCC or PGL before 50 years of age with/without multifocal disease (9). In the past, detecting a variant (either germline or somatic) associated with the development of disease involved the testing of sequential single genes, as predicted by clinical and morphological feature-driven diagnostic algorithms, individualized according to the likelihood of a syndromic cause, and informed decisions made with the patient (1,5,19,21). However, this testing protocol remained expensive and extremely time-consuming (17). For example, in 2012 the cost involved to test a specific patient for the *SDHB*, *SDHC*, *SDHD*, *VHL* and *RET* genes was approximately $4100 (3). Also, because the number of affected genes continues to grow, so does the complexity of genetic testing (3,9,27). In individuals in whom a genetic aetiology is suspected, next-generation sequencing allows a targeted approach to massive parallel sequencing of all genes of interest in a single, accurate, cost-effective and timely run compared to conventional Sanger sequencing which has been used for many years (10,18,28).

Next-generation sequencing has the added benefit in that it can be applied to formalin-fixed, paraffin-embedded tissue (14). Various proposed genetic testing algorithms have been formulated to aid in genetic testing in any patient diagnosed with a PCCs or PGLs, to assist with an effective and thorough, yet cost-effective medical work-up (18). Now it is both practical and cost-effective to offer genetic testing to all cases of young age-onset and/or metachronous or synchronous disease (5). Also, when biomarkers for response and/or resistance to therapy emerge, next-generation-sequencing analysis can aid in clinical decisions regarding patient follow-up and the necessary treatment adjustment (14).

Currently, some laboratories in America have developed a PCC and PGL testing panel where mutations in thirteen susceptibility genes are detected by using next-generation sequencing technology (17).

However, the implementation of next-generation-sequencing is certainly challenging, since this technology generates an extremely large amount of sequence data, which makes the interpretation of the results very complex (17). It is therefore strongly advised, that the final report of any next-generation-sequencing test should be a multidisciplinary effort with contributions from geneticists and pathologists, combined with an exhaustive literature review to determine and classify the genetic variants discovered (17).

Internationally, extensive research has been done and information obtained regarding the genetic make-up of PCCs and PGLs (18), even though the differential expression of susceptibility genes in different races and ethnicities have not been well documented (29). On the local front, the available data for our population is severely lacking. To date, there has been no research done regarding the specific gene mutations associated with PCCs and PGLs in the South African population, and especially in South African black patients.

## AIM AND OBJECTIVES

*Aim*

To describe the profile of variants in PCCs/PGLs in the South African population.

*Objectives*

To determine the following:

- Whether any variants are present in the South African population presenting with PCCs/PGLs, which have not yet been described internationally.
- Whether one can formulate an order of gene testing specific for the South African population, to facilitate genetic diagnosis in a cost-effective but highly sensitive manner.

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**CHAPTER 2**

## ARTICLE: THE PROFILE OF GENETIC MUTATIONS IN PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS IN SOUTH AFRICAN PATIENTS

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The article was prepared according to the journal submission guidelines for the *American Journal of Surgical Pathology* (cf. Appendix D).

**THE PROFILE OF GENETIC MUTATIONS IN PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS IN SOUTH AFRICAN PATIENTS**

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**ABSTRACT**

Pheochromocytomas (PCCs) are rare neoplasms of the adrenal glands while extra-adrenal pheochromocytomas are referred to as paragangliomas (PGLs). Both germline and somatic variants are associated with their pathogenesis, and identification of a specific variant guides clinical management in patients and their families. The genetic heterogeneity of PCCs/PGLs is distinct, creating the possibility of personalized, genomics-driven therapy. Next-generation sequencing allows massive parallel sequencing of all genes of interest in a single, cost-effective run. Internationally, extensive research has been done regarding the genetic make-up of PCCs/PGLs, with the genes most commonly involved being *SDHB, SDHD, RET,* and *VHL*. The aim of this study was to describe the genetic variants in PCCs/PGLs in the South African population. A retrospective study was performed which included the 98 most recent cases with sufficient tissue available in wax blocks. Manual DNA extraction and subsequent DNA sequencing was performed, and 16 genes of interest were assessed for possible variants. Thirty one of the 98 cases were sequenced. The DNA quality was inadequate in the remaining cases. Ninety three variants were detected with 32 synonymous variants, 54 missense variants, and 7 truncating variants. Nine pathogenic or likely pathogenic variants were identified in total, involving the *NF1*, *KIF1B*, *RET, SDHB* and *TMEM127* genes. Most variants identified were predicted benign, likely benign and benign variants or variants of unknown significance. The profile of pathogenic and likely pathogenic variants identified in this study differs from that found in the international literature. However, the number of patients currently evaluated is small and additional data are required for an accurate local genetic profile.

**Keywords:** Pheochromocytoma, paraganglioma, next-generation sequencing, genetic variants, germline, sporadic, familial, syndromic.

## INTRODUCTION

Pheochromocytomas (PCCs) are rare neoplasms of the adrenal medulla with similar neoplasms originating at extra-adrenal sites referred to as paragangliomas (PGLs); the two most common extra-adrenal sites being the carotid body and the jugulo-tympanic apparatus of the middle ear (5). The mean age at diagnosis is the fourth and fifth decades, although familial cases tend to occur in a slightly younger age group, and about 10% of cases affect the paediatric population (7). Males and females are roughly equally affected (5), although head-and-neck paragangliomas are more frequently associated with a female predominance, which is even more pronounced at higher altitudes, in which case the female-to-male ratio is 8:1 (8). Patients with functional tumours usually present with a characteristic triad of sweating attacks, tachycardia and headaches related to catecholamine hypersecretion (9). The patients also commonly suffer from severe hypertension of abrupt onset, which in some cases is unresponsive to treatment (2,7,10).

The incidence of these tumours ranges from 1 per 2500 to 1 per 6500 people (13). This wide variation is probably due to the fact that these tumours are quite often missed, and in many instances only diagnosed post-mortem (7).

PCCs and PGLs occur in all races but have mainly been described in Caucasians. Research relating to PCCs and PGLs in African patients is limited and consists mainly of single cases and small series (30), that reported a similar incidence, age and sex distribution, symptomatology, macroscopic appearance, histology and complications, to that seen in Western countries (15). More recently, the similarities between Caucasian and African patients have been confirmed in a 30-year South-African audit done between 1980 and 2009 which included 54 black patients (11).

Up to 40% of PCCs and PGLs are inherited (1,5,9,14,16). PCCs and PGLs can be classified as syndromic, familial or sporadic and both germline and somatic mutations are associated with its tumorigenesis (14). The most common familial syndromes known to be associated with PCCs and PGLs include Multiple Endocrine Neoplasia (MEN) type 2A/B, Neurofibromatosis (NF) type 1, Von Hippel-Lindau disease and Familial Paraganglioma Syndromes 1-5 (5,9,13,16,19).

PCCs and PGLs have a marked genetic heterogeneity and multiple genes have been confirmed to participate in the pathogenesis of PCCs and PGLs, with new ones added annually

(1,3,10,13,17,19,21,24–26). Three classes of major susceptibility genes, each with distinct molecular profiles have been described (2,5,14,17,18,21,22,27). Cluster 1 genes are associated with the pseudo-hypoxic signature and overexpression of angiogenesis factors, and mainly correspond to SDH- and VHL-related tumours. They are divided into cluster 1A genes which include *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MDH2*, and *FH* and cluster 1B genes which include *VHL*, *EPAS1* (*HIF2A*), *ELGN1* (*PHD2*) and *IDH1*. Cluster 2 genes, predominantly *RET* and *NF1* mutations, are associated with increased activation of kinase signalling pathways RAS/RAF/MAPK and PI3K/AKT/mTOR and include *RET*, *NF1*, *KIF1Bß*, *MAX* and *TMEM127*.

Other genes are also implicated in the development of PCCs and PGLs, but their exact role in the pathogenesis is yet to be established. These genes include *GDNF*, *HRAS*, *KRAS*, *GNAS*, *CDKN2A*, *TP53*, *BAP1*, *BRCA1*, *BRCA2*, *ATRX*, *KMT2D*, *HIF2*, *SLC25A11*, *GOT2*, *DNMT3A*, *BRAF*, *TERT*, *MET*, *CDH1*, *KMT2D*, *FHIT* and *MEN1*.

The genes most commonly involved according to international literature are *SDHB*, *SDHD*, *RET* and *VHL* (9). Internationally, extensive research has been done and information obtained regarding the genetic make-up of PCCs and PGLs (18), even though the differential expression of susceptibility genes in different races and ethnicities have not been well documented (29). On the local front, the available data for our population is severely lacking. To date, there has been no research done regarding the specific gene mutations associated with PCCs and PGLs in the South African population, and especially in South African black patients.

The aim of this project was therefore to describe the profile of mutations in PCCs and PGLs in the South African population including any new mutations which have not yet been described internationally, using next-generation sequencing. Using this data, it might be possible to develop an order of gene testing specifically for the South African population, to facilitate genetic diagnosis in a cost-effective but highly sensitive manner.

## METHODOLOGY

### Specimen selection

A retrospective study was performed. Cases histologically confirmed as PCCs/PGLs in the period between January 2005 and November 2017 at the Department of Anatomical Pathology, the University of the Free State and National Health Laboratory Service, Bloemfontein, South Africa were evaluated for possible inclusion in the study. Cases were identified by performing a search on the DISA and LabTrak laboratory information systems for the period January 2005 to November 2017 using the appropriate SNOMED codes. Subsequently, the corresponding slides and wax blocks were retrieved from the departmental archives and the slides were evaluated to confirm the initial diagnosis.

The 98 most recent cases with sufficient available tissue in the wax blocks were included in the study. Demographic data including age, sex, race, and location of the tumour was obtained from the pathology reports. Any cases with insufficient or unavailable tissue in the wax blocks were excluded from the study. Approval to perform the study was obtained from the Health Sciences Research Ethics Committee of the University of the Free State (UFS-HSD2017/1430).

### Laboratory procedure

Sections of formalin-fixed, paraffin-embedded (FFPE) tissue of approximately 20µm in thickness were placed into microcentrifuge tubes. The paraffin was removed with xylene and washed with ethanol. DNA extraction was performed manually according to the manufacturer's instructions using the QIAamp DNA FFPE protocol (Qiagen, Valencia, CA). The obtained DNA samples were sent to the DNA Sequencing Unit of the Central Analytical Facilities at the University of Stellenbosch. Before commencing with the sequencing-library preparation, quality control was performed on the DNA samples to determine whether they were suitable for further processing. Since the DNA was extracted from FFPE specimens, it was important to establish that the DNA was not too fragmented. The subsequent steps involved library preparation using a custom ordered HaloPlex kit (Agilent) which was also used for target enrichment. During the library preparation, molecular barcodes were added to differentiate between the different specimens. Quality control was again performed on the sequencing-library. Finally, the library was loaded onto the Ion 530 Chip and sequencing was performed using the Ion S5 sequencing System (ThermoFisher Scientific).

Variant calling or single nucleotide polymorphisms (SNP) detection was performed using well-cited software. In brief, a quality analysis of the read data was performed using Prinseq. Data passing the quality step was mapped to the human chromosomes where those targets reside. This step, short read mapping, was done using Borrows Wheel Aligner's MEM algorithm. The necessary file conversions and read depth calculation were done with Samtools. VarScan2 and Freebays were used to filter and score SNPs based on Heuristics and Bayesian statistics. The resulting variant call files (vcf) was used in conjunction with online databases, such as ENSEMBLE where various predictive algorithms were used to interpret the resulting phenotype. These also contained population statistics for known variants.

During the sequencing process, the following genes were assessed for possible variants: *EGLN1*, *EPAS1*, *FH*, *HRAS*, *IDH1*, *KIF1B*, *MAX*, *NF1*, *RET*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *TMEM127* and *VHL*.

**RESULTS**

Due to suboptimal DNA quality, only 31 of the 98 cases included in the study were sequenced. Of the 31 cases, 21 patients (67.8%) were female, 9 patients (29%) were male and in 1 case (3.2%) the sex was not specified. Twenty-two patients (71%) were Black, five (16.1%) were Caucasian and 4 patients (12.9%) were Coloured. The mean age of the patients was 47.2 years with a median age of 42.5 years and an age range of 7 to 78 years. The age distribution of the cases is shown in Table 1.

**Table 1. Age distribution**

| **Age** | **Number of patients** |
|---|---|
| <20 years | 1 (3.2%) |
| 20-29 years | 3 (9.7%) |
| 30-39 years | 5 (16.1%) |
| 40-49 years | 10 (32.3%) |
| 50-59 years | 6 (19.4%) |
| 60-69 years | 3 (9.7%) |
| >69 years | 3 (9.7%) |
| Total | 31 |

The carotid body and middle ear were the two most common locations, with 11 cases (35.5%) presenting in the carotid body and 8 cases (25.8%) presenting in the middle ear. The tumour location of the cases is shown in Table 2.

**Table 2. Tumour location**

| **Location of tumour** | **Number of patients** |
|---|---|
| Carotid body | 11 (35.5%) |
| Middle ear | 8 (25.8%) |
| Adrenal gland | 2 (6.5%) |
| Organ of Zuckerkandl | 2 (6.5%) |
| Cervical Lymph Node Metastasis | 3 (9.8%) |
| Other | 5 (16.1%) |
| Total | 31 |

Of the 16 genes that were sequenced, 93 variants were detected with 32 synonymous variants, 54 missense variants and 7 truncating variants. A synonymous variant implies a nucleotide change was present, but with no subsequent amino acid translational change. The majority of these variants are benign, or likely benign. A missense variant implies a nucleotide change was present that subsequently translated into a different amino acid. The majority of these variants are of unknown clinical significance. A truncating variant implies a premature stop in translation was present that resulted in an incomplete and usually nonfunctional protein product. The majority of these variants are pathogenic, or likely pathogenic. All 31 patients had at least one variant. A breakdown of the variants that were identified is summarized in Table 3.

**Table 3. Summary of variants detected**

| Gene | Synonymous variants | | | | | Missense variants | | | | | Truncating variants | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B/LB | VUS | | | LP/P | B/LB | VUS | | | LP/P | LP/P | |
| | | PB | U | PP | | | PB | U | PP | | | |
| *EGLN1* | 1 | | 1 | | | 2 | | | | | | 4 |
| *EPAS1* | 4 | | 2 | | | 2 | | 1 | 1 | | | 10 |
| *FH* | 2 | | 1 | | | 2 | | | 1 | | | 6 |
| *IDH1* | 1 | | | | | | | | | | | 1 |
| *KIF1B* | 2 | | 1 | | | 2 | 3 | 2 | 9 | | 2 | 21 |
| *MAX* | | | | | | | | 1 | | | | 1 |
| *NF1* | 6 | | 1 | | | | 3 | 2 | 4 | | 2 | 18 |
| *RET* | 4 | | | | | | 2 | 1 | 1 | 1 | 1 | 10 |
| *SDHA* | 1 | | 1 | | | 2 | 1 | 1 | 2 | | | 8 |
| *SDHAF2* | 1 | | | | | | | | | | | 1 |
| *SDHB* | 1 | | | | | 1 | 1 | | | 1 | 1 | 5 |
| *SDHC* | | | | | | | | 1 | | | | 1 |
| *SDHD* | 1 | | | | | 1 | 1 | | | | | 3 |
| *TMEM127* | 1 | | | | | | | | 1 | | 1 | 3 |
| *VHL* | | | | | | 1 | | | | | | 1 |
| **Total** | **32** | | | | | **54** | | | | | **7** | **93** |

B = benign; LB = likely benign; VUS = variant of unknown significance; U = unknown significance; PP = predicted pathogenic; LP = likely pathogenic; P = pathogenic.

The clinical significance of the variants is depicted in Table 4.

**Table 4. Clinical significance of the variants**

| | | |
|---|---|---|
| B | Benign | > 95% chance of being associated with benign biological behaviour |
| LB | Likely Benign | > 80% chance of being associated with benign biological behaviour |
| VUS | Variant of unknown significance | Not enough evidence or evidence inconclusive or significantly contradicting |
| PB | Predicted Benign | > 60% chance of being associated with benign biological behaviour |
| U | Unknown significance | |
| PP | Predicted Pathogenic | >60% chance of being associated with pathogenic biological behaviour |
| LP | Likely pathogenic | >80% chance of being associated with pathogenic biological behaviour |
| P | Pathogenic | >95% chance of being associated with pathogenic biological behaviour |

Nine pathogenic or likely pathogenic variants were identified; two involving the *NF1* gene, two involving the *KIF1B* gene, two involving the *RET* gene, two involving the *SDHB* gene, and one variant involving the *TMEM127* gene. Seven were truncating variants and 2 were missense variants. Table 5 highlights the specific genetic variant together with the patient characteristics.

**Table 5. Pathogenic or likely pathogenic variants with associated patient characteristics**

| Sample | Patient age | Patent race | Patient sex | Tumour location | Diagnosis and known clinical associations | Gene and variant |
|---|---|---|---|---|---|---|
| 3 | 52 | Black | Male | Left carotid body | Paraganglioma<br><br>None | ***NF1***<br><br>Truncation |
| 6 | 28 | Black | Female | Right pectoralis / deltoid soft tissue mass | Paraganglioma; most likely metastatic<br><br>Known with a large retroperitoneal mass | ***SDHB***<br><br>Missense |
| 7 | 33 | Coloured | Male | Right adrenal mass | Pheochromocytoma<br><br>Possible MEN2A syndrome | ***RET***<br><br>Missense |
| 14 | 49 | White | Male | Left submandibular lymph node | Paraganglioma; metastatic<br><br>Known with Neurofibromatosis | ***SDHB***<br><br>Extension<br>Truncation<br>Site |
| 19 | 23 | Black | Male | Organ of Zuckerkandl | Paraganglioma<br><br>None | ***NF1***<br><br>Truncation |
| 23 | 50 | Coloured | Female | Right intravagal nerve | Paraganglioma<br><br>None | ***KIF1B***<br><br>Truncation |
| 68 | 28 | Black | Female | Left carotid body | Paraganglioma<br><br>None | ***TMEM127***<br><br>Truncation |
| 76 | 76 | Black | Female | The carotid body (Laterality not stated) | Paraganglioma<br><br>None | ***KIF1B*** Truncation<br>***RET*** Truncation |

An additional nineteen predicted pathogenic missense variants were identified; one involving the *EPAS1* gene, one involving the *FH* gene, nine involving the *KIF1B* gene, four involving the *NF1* gene, one involving the *RET* gene, two involving the *SDHA* gene, and one variant involving the *TMEM127* gene.

There were, therefore, a total of twenty-eight predicted pathogenic, likely pathogenic and pathogenic genetic variants. A summarized overview is depicted in Table 6.

**Table 6. Predicted pathogenic, likely pathogenic and pathogenic genetic variants**

| **Gene** | **Predicted Pathogenic** | **Likely Pathogenic / Pathogenic** | | **% of the variants depicted** |
|---|---|---|---|---|
| | *Missense variants* | *Missense variants* | *Truncating variants* | |
| *EPAS1* | 1 | | | 3.6% |
| *FH* | 1 | | | 3.6% |
| *KIF1B* | 9 | | 2 | 39.3% |
| *NF1* | 4 | | 2 | 21.4% |
| *RET* | 1 | 1 | 1 | 10.7% |
| *SDHA* | 2 | | | 7.1% |
| *SDHB* | | 1 | 1 | 7.1% |
| *TMEM127* | 1 | | 1 | 7.1% |
| **Total** | **19** | **9** | | |

## DISCUSSION

This study is the first to describe genetic variants associated with PCCs and PGLs in the South African population. The most common genes associated with PCCs and PGLs according to international literature are *SDHB*, *RET*, *SDHD* and *VHL*, with hospital- and population-based series citing an incidence of between 19 and 24% (9). In order of decreasing incidence, *SDHD* variants were found in 8% of cases, *VHL* variants in 6% of cases, *SDHB* variants in 4% of cases and *RET* variants in <1% of cases (4). This differs from the profile of genetic variants in this study, as although some variants were found in the *SDHB* and *RET* genes, with 7.1% and 10.7% respectively, no *SDHD* or *VHL* variants were identified (taking in consideration only predicted pathogenic, likely pathogenic and pathogenic variants). Most of the variants identified were *KIF1B*, *NF1* and *RET* variants with 39.3%, 21.4% and 10,7% respectively. Interestingly, in one patient, two pathogenic or likely pathogenic variants were identified, involving the *RET* and *KIF1B* genes.

The *KIF1B* gene is located on chromosome 1p36.22, and deletion of this region is frequently associated with neural crest-derived tumours (2,17,31). The *KIF1B* gene encodes two protein isoforms, namely *KIF1Bα* and *KIF1Bβ*. Specifically, *KIF1Bβ* acts as a tumour suppressor mediating neuronal apoptosis (2,17,31). A specific syndrome has not been yet associated with *KIF1B* mutations, but germline mutations have been documented in patients with PCCs, ganglioneuromas, neuroblastomas and lung carcinomas (2,17,31).

The *NF1* gene is located on chromosome 17q11.2 and encodes the protein neurofibromin which is mainly expressed in the nervous system where it inhibits cellular proliferation, and germline mutations are associated with the clinical syndrome of neurofibromatosis type 1 (2,17,31). PCCs are seen in approximately 0.1%-5.7% of patients with neurofibromatosis type 1 (2,17,31).

The *RET* gene is located on chromosome 10q11.21 and acts as a proto-oncogene involved in the activation of various intracellular pathways involved in cellular growth and differentiation (2,17,31). An activating mutation of the *RET* gene underlies the clinical syndromes of MEN2A and MEN2B, both of which have approximately a 50% risk of developing a PCC (2,17,31).

Twenty of the 28 (75%) predicted pathogenic, likely pathogenic and pathogenic variants identified were missense variants while remaining 7 variants (25%) were truncating variants. The majority of truncating variants are likely pathogenic or pathogenic as they result in a non-functional protein product.

Unfortunately, a major limiting factor in this study was the suboptimal nature of the DNA material extracted from the paraffin-embedded, formalin-fixed tissue with only 31 of the 98 selected cases being of adequate quality to perform next-generation sequencing. This is partly due to the formalin fixation as formalin is known to cause time-dependant fragmentation and degradation of the DNA strands. Also, many of the samples submitted were from referral laboratories and the composition and/or the quality of the formalin that was utilised initially was sometimes of poor quality or not adequality buffered, all of which harms the overall DNA structural integrity. Whether the resultant small sample size is adequate to extrapolate the results obtained to the South African population in general, is open for debate. The limited number of pathological variants identified, from our even more limited sample size, hampers the formulation of a testing algorithm. However, it suggests that single-gene testing for the *KIF1B* and *NF1* genes can be considered first, followed by the *RET* gene. As immunohistochemistry for *SDHA* and *SDHB* is available in our laboratory, screening for *SDH* can initially be conducted with this method and any cases with a loss of staining can then be referred for sequencing.

Another challenge associated with any research that involves next-generation sequencing technology is the fact that it generates large amounts of sequence data, which makes the interpretation of the results very complex. It is therefore strongly advised that the final report of any next-generation-sequencing test should be a multidisciplinary effort with contributions from geneticists and pathologists, combined with an exhaustive literature review to determine and classify the genetic variants discovered (17). As clearly illustrated in this study, most variants identified were predicted benign, likely benign and benign variants or variants of unknown significance. Sixty-five of the 93 (70%) variants identified in this study fell in these categories.

## CONCLUSION

Identification of a specific gene variant is of the utmost importance because it guides clinical management in variant-positive patients and their families in terms of accurate diagnosis, prognosis, family screening and planning therapeutic options (1,4,9,16,20,21). Thus, offering overall improved care for patients and their families.

With next-generation sequencing technology, it is now both practical and cost-effective to offer genetic testing to all patients with a PCC or PGL with a young age at onset and/or metachronous or synchronous disease. Also, when biomarkers for response and/or resistance to therapy emerge, next-generation-sequencing analysis can aid in clinical decisions regarding patient follow-up and the necessary treatment adjustment.

The activated molecular pathways of PCCs and PGLs create the possibility of future personalized, genomics-driven therapy based on the underlying mutation, as well as the determination and implementation of diagnostic and prognostic biomarkers of malignancy useful in overall patient care (10,14). This molecular-based therapy would comprise of antiangiogenic drugs for Cluster 1 tumours and mTOR or RAS-RAF pathway inhibitors for tumours belonging to Cluster 2. Continued use of next-generation sequencing-based assays including the less commonly involved genes, will improve our knowledge of the mutational burden and associated phenotypes of PCCs and PGLs (22).

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## APPENDIX A

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HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

06 December 2017

L MAREE
DEPT OF ANATOMICAL PATHOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear L Maree

**HSREC 155/2017 (UFS-HSD2017/1430)**
**PRINCIPAL INVESTIGATOR: L MAREE**
**PROJECT TITLE: THE PROFILE OF GENETIC MUTATIONS IN PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS IN SOUTH AFRICAN PATIENTS**

**APPROVED**

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved this project at the meeting held on 05 December 2017 after all conditions were met.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

.....

PROF WJ STEINBERG
VICE CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
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**APPENDIX B**

UNIVERSITY OF THE FREE STATE
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UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

09th October 2017

Heath Sciences Research Ethics Committee
Faculty of Heath Sciences
University of the Free State.

Dear Chair

I grant permission for the following

Anatomical Pathology Registrar: **Dr L Maree**
(Supervisor: Prof J Goedhals)

to conduct the following **MMed study** within the School of Pathology:
"The profile of genetic mutations in phaeochromocytomas and paragangliomas in South African patients"

I wish Dr Maree much success in her study.

Yours Sincerely

Jocelyn Naicker
Head: School of Pathology
Faculty of Health Sciences
University of the Free State
Tel: 051 405 2914 | Cell: 0829071925
jocelyn.naicker@nhls.ac.za | www.nhls.ac.za

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**APPENDIX C**

# THE PROFILE OF GENETIC MUTATIONS IN PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS IN SOUTH AFRICAN PATIENTS

## RESEARCHERS


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## INTRODUCTION

Pheochromocytomas (PCCs) are rare, usually benign, neoplasms composed of chromaffin (neuro-endocrine) cells. Two-thirds of these tumours are functional and synthesize and release catecholamines. Extra-adrenal PCC's are referred to as paragangliomas (PGLs). The two most common sites for PGL's are the carotid body and the jugulo-tympanic apparatus of the middle ear. The mean age of diagnosis is the fourth to fifth decades, although familial cases tend to occur slightly earlier, and about 10% of cases affect the paediatric population. Males and females are equally affected. The traditionally used 'rule-of-tens' (10% familial, 10% malignant, 10% extra-adrenal) no longer holds. The current conclusion is that up to 30% of PCCs are of inherited origin, about 15% occur in extra-adrenal locations and the frequency of malignancy ranges from 3-36%, where metastases can occur up to twenty years after removal of the primary tumour.

The incidence of these tumours ranges between 1 per 100,000-300,000, but this may be an underestimate. This non-specific value is probably due to the fact that these tumours are quite often missed, and in many instances only diagnosed post-mortem. This may be due to a low index of suspicion on the physicians' side, the non-specific nature of the symptoms, as well as the fact that approximately 30% of these tumours are non-functional.

Despite the fact that these tumours are quite rare, prompt clinical detection is of the utmost importance since they are potentially lethal. This is due to their ability to secrete catecholamines and their potentially malignant behaviour. PCCs and PGLs occur in all races, but have mainly been described in Caucasians. Research relating to PCCs and PGLs in African patients is limited and consists mainly of single cases and small series [1,2,] that reported a similar incidence, age and sex distribution, symptomatology, macroscopic appearance, histology and complications, to that seen in Western countries. More recently, the similarities between Caucasian and African patients was confirmed in a 30-year South-African audit [3] done between 1980 and 2009 which included 54 black patients. The lack of data from the African continent as a whole is probably due to limited health resources in general, as well as limited laboratory and radiological facilities to aid in the diagnosis of PCCs.

Both germline and somatic mutations are associated with the development of PCCs. A germline mutation is inherited through the germ cells and is therefore present in every cell in the body and will be passed on to future generations. In contrast, a somatic mutation is acquired after fertilisation, is only present in the tumour cells and is not inherited by any offspring. As stated earlier, up to 30% of PCCs are associated with germline mutations and are then usually seen in the context of a specific familial syndrome. The familial syndromes, with their associated germline-mutations known to be involved in the tumorigenesis of PCCs, are as follows [4]:

| Syndrome | Gene |
|---|---|
| Multiple endocrine neoplasia type 2A | RET |
| Multiple endocrine neoplasia type 2B | RET |
| Neurofibromatosis type 1 | NF1 |
| Von Hippel-Lindau | VHL |
| Familial paraganglioma 1 | SDHD |
| Familial paraganglioma 2 | SDHDF2 |
| Familial paraganglioma 3 | SDHC |
| Familial paraganglioma 4 | SDHB |
| Familial paraganglioma 5 | SDHA |

A small fraction of PCCs are associated with other syndromes, including Carney triad, Carney Stratakis syndrome and very rarely, MEN1.

Identification of a specific gene mutation/s is important because it can guide medical management in mutation-positive patients and their families [5,6], because:

- patients should be investigated for the presence of other supra- and infra-diaphragmatic PCCs,
- patients should be investigated for the presence of other associated tumours in a patient with a syndromic disorder,
- patients with a germ-line mutation have an overall poor prognosis and require close and regular follow-up to monitor for any recurrences or malignant transformation,
- diagnosis of hereditary PCCs also allows genetic screening in first-degree relatives of the index subject,

This will then lead to early diagnosis, treatment, regular surveillance and better prognosis for patients and their relatives. In the future personalized therapy based on the underlying mutation may also be an option.

A distinct feature of PCCs is their genetic heterogeneity and to date 24 genes have been confirmed to participate in PCC tumorigenesis [7, 8, 9, 10, 11, 12, 13], which falls into three broad classes:

- Cluster 1 mutations associated with the pseudohypoxic pathway:

- PHD2, VHL, SDHA, SDHB, SDHC, SDHD, SDHF2, IDH, HIF2A, MDH2, FH

- Cluster 2 mutations associated with altered activation of kinase signalling pathways:

- RET, NF1, KIF1Bß, MAX, TMEM127, MEN1

- Other genes also implicated in the development of PCCs:

- GDNF, HRAS, KRAS, GNAS, CDKN2A (p16), p53, BAP1, BRCA1&2, ATRX, KMT2D

The gene most commonly mutated is SDHB. In the past, detecting a mutation (either germ-line or somatic) associated with the development of PCCs involved the testing of single genes, as predicted by clinical and morphological characteristics. However, this testing protocol was expensive and extremely time consuming. Also, because the number of affected genes continues to grow, so does the complexity of genetic testing. Next-generation sequencing allows simultaneous sequencing of various genes in a single, accurate, cost-effective and timely run [14]. A proposed genetic testing algorithm has been formulated [15] to aid in genetic testing in any patient diagnosed with a PCC/PGL, with the intention of assisting with an effective and thorough, yet cost-effective medical work-up.

Internationally, abundant research has been done and information obtained regarding the genetic make-up of PCCs/PGLs. However, on the local front the available data for our population is severely lacking. To date there has been no research done regarding the specific gene mutations associated with PCCs/PGLs in the South African population, and especially in South African black patients.

## AIM

To describe the following:

- The profile of mutations in PCCs/PGLs in the South African population.

## OBJECTIVES

To determine the following:

- Whether any mutations are present in the South African population presenting with PCCs/PGLs, which have not yet been described internationally.
- Whether one can formulate an order of gene testing specific for the South African population, to facilitate genetic diagnosis in a cost-effective but highly sensitive manner.

## METHODOLOGY

### Specimen selection

A retrospective study will be performed. Cases histologically confirmed as PCCs/PGLs in the period between January 2005 and December 2016 at Universitas Academic Laboratories, NHLS, Bloemfontein, will be evaluated for possible inclusion in the study. Cases will be identified by performing a computer search on DISA and LabTrak for the period 2005 – 2016 using SNOMED codes. Slides and wax blocks will be retrieved from the Department of Anatomical Pathology archives accordingly. The slides will be evaluated by Dr Maree and Prof Goedhals to confirm the diagnosis. The most recent 96 cases with sufficient available tissue in wax blocks will be used as this is the number of specimens which can be processed using the HaloPlex kit as described under laboratory procedure. Demographic data including age, sex, race and site of the tumour will be obtained from the pathology reports.

Each patient will be given a study number and no patient names will be used. Any cases with insufficient or unavailable tissue in the wax blocks will be excluded from the study.

### Laboratory procedure

Sections of formalin fixed, paraffin embedded tissue of approximately 20µm in thickness will be placed into micro centrifuge tubes. The paraffin will be removed with xylene and washed with ethanol. DNA extraction will be performed manually according to the manufacturer's instructions using the QIAamp DNA FFPE protocol. The obtained DNA will be sent to the Central Analytical Facilities (University Stellenbosch), specifically to the DNA sequencing Unit. Before commencing with the sequencing-library preparation, quality control will be performed on the DNA samples in order to determine whether they are suitable for further processing. Since the DNA is extracted from FFPE specimens it is important to establish that the DNA is not too fragmented. The subsequent steps involve library preparation using a custom ordered HaloPlex kit (Agilent) which is also used for target enrichment. During library preparation molecular barcodes are added to differentiate between the different specimens. Quality control is again performed on the sequencing-library. Finally the library is loaded onto the Ion 530 Chip and sequencing is performed using the Ion S5 sequencing System (ThermoFisher Scientific).

Variant calling or SNP (single nucleotide polymorphisms) detection will be performed using well cited software. In brief, quality analysis of the read data will be performed using prinseq. Data passing the quality step will be mapped to the human chromosomes where these targets reside. This step, short read mapping, will be done using Borrows Wheel Aligner's MEM algorithm. The necessary file conversions and read depth calculation will be done with

samtools. VarScan2 and freebays will be used to filter and score SNPs based on heuristics and Bayesian statistics. The resulting vcf (variant call files) can be used with online databases, such as ENSEMBLE where various predictive algorithms are used to interpret the resulting phenotype. These also contain population statistics for known variants.

During the sequencing process the following genes will be assessed for possible mutations: **EGLN1, EPAS1, FH, HRAS, IDH1, KIF1B, MAX, NF1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127, VHL.**

## TIME SCHEDULE

It should take approximately 18 months to complete the study. This will allow 12 months for laboratory work and data collection and 6 months for analysis and write up.

## DATA ANALYSIS

Information will be entered into an Excel worksheet and analysed by the researchers under the supervision of Mr A Bester. Results will be summarised by frequencies and percentages (categorical variables) and means, standard deviations or percentiles (numerical variables). Subgroups will be compared using relative risks or 95% confidence intervals for differences in percentages, means or medians.

## BUDGET

| | |
|---|---|
| QIAmp DNA FFPE tissue kit x 2 | R12 060.21 |
| HaloplexHS 1-250kb, ION, 96 | R233 166.48 |
| Sequencing costs | R140 000.00 |
| **Total** | **R385 226.69** |

The costs are being covered by funds from the Department of Surgery.

## ETHICS

Each case will be allocated a unique study number. Molecular testing and data analysis will be performed using only the unique study number in order to protect the participants' identities. All data will be treated with strict confidentiality. Permission to perform the study has been obtained from Dr J Naicker, Head of the School of Pathology, NHLS and University of the Free State. As only wax blocks and information from the pathology reports will be used it will not be necessary to obtain approval from the Free State Department of Health. This project represents a sub-study of a project for which ethics approval has already been obtained namely 'Characteristics of Paragangliomas/Pheochromocytomas in patients presenting at Anatomical Pathology, Universitas Academic Laboratories' (HREC NR 55/2016)

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# APPENDIX D



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It is the author's responsibility to ensure that a patient's anonymity be carefully protected and to verify that any experimental investigation with human subjects reported in the manuscript was performed with informed consent and following all the guidelines for experimental investigation with human subjects required by the institution(s) with which all the authors are affiliated. Authors should mask patients' eyes and remove patients' names from figures unless they obtain written consent from the patients and submit written consent with the manuscript.

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Authors must state all possible conflicts of interest in the manuscript, including financial, consultant, institutional and other relationships that might lead to bias or a conflict of interest. If there is no conflict of interest, this should also be explicitly stated as none declared. All sources of funding should be acknowledged in the manuscript. All relevant conflicts of interest and sources of funding should be included on the title page of the manuscript with the heading "Conflicts of Interest and Source of Funding:". For example:

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Authors should read and follow the ICMJE general guidelines for conducting and reporting medical studies, as well as the specific guidelines outlined here for AJSP submissions. http://www.icmje.org/icmje-recommendations.pdf

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Include on the title page (a) complete manuscript title; (b) authors' full names, highest academic degrees, and affiliations; (c) name and address for correspondence, including fax number, telephone number, and e-mail address; (d) address for reprints if different from that of corresponding author; and (e) all sources of support, including pharmaceutical and industry support, that require acknowledgment.

The title page must also include disclosure of funding received for this work from any of the following organizations: National Institutes of Health (NIH); Wellcome Trust; Howard Hughes Medical Institute (HHMI); and other(s).

**Unstructured Abstract and Key Words**

**Abstract must be submitted as a separate file.** Limit the abstract to 250 words. It must be factual and comprehensive. Limit the use of abbreviations and acronyms, and avoid general statements (eg, "the significance of the results is discussed"). List three to five key words or phrases.

**Text**

Organize the manuscript into four main headings: Introduction, Materials and Methods, Results, and Discussion. References and figure legends should be placed after the Discussion section. Define abbreviations at first mention in text and in each table and figure. If a brand name is cited, supply the manufacturer's name and address (city and state/country).

**Abbreviations**

For a list of standard abbreviations, consult the Council of Biology Editors Style Guide (available from the Council of Science Editors, 9650 Rockville Pike, Bethesda, MD 20814) or other standard sources. Write out the full term for each abbreviation at its first use unless it is a standard unit of measure.

**References**

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*Journal article*

1. Band NS, Dawson JM, Juliao SF, et al. In vivo macrophage recruitment by murine intervertebral disc cells. *J Spinal Disord.* 2001;14:339--342.

*Book chapter*

2. Crowe VR. Visual information analysis: frame of reference for visual perception. In: Kramer P, Hinojosa J, eds. *Frames of Reference for Pediatric Occupational Therapy*. Philadelphia, PA: Lippincott Williams & Wilkins; 1999:205–256.

*Entire book*
3. Dellman RM, Marentette LJ. *Atlas of Craniomaxillofacial Fixation*. Philadelphia, PA: Lippincott Williams & Wilkins; 1999.

*Software*
4. *Epi Info* [computer program]. Version 6. Atlanta, GA: Centers for Disease Control and Prevention; 1994.

*Online journals*
5. Friedman SA. Preeclampsia: a review of the role of prostaglandins. Obstet Gynecol [serial online]. January 1988;71:22-37. Available from: BRS Information Technologies, McLean, VA. Accessed December 15, 1990.

*Database*
6. G-PDQ [database online]. Bethesda, MD: National Cancer Institute; 1996. Updated March 29, 1996.

*World Wide Web*
7. Hostin LO. Drug use and HIV/AIDS [JAMA HIV/AIDS Web site]. June 1, 1996. Available at: http://www.ama-assn.org/special/hiv/ethics. Accessed June 26, 1997.

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3. Upload each figure to Editorial Manager in conjunction with your manuscript text and tables.

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- Number figures in the figure legend in the order in which they are discussed.
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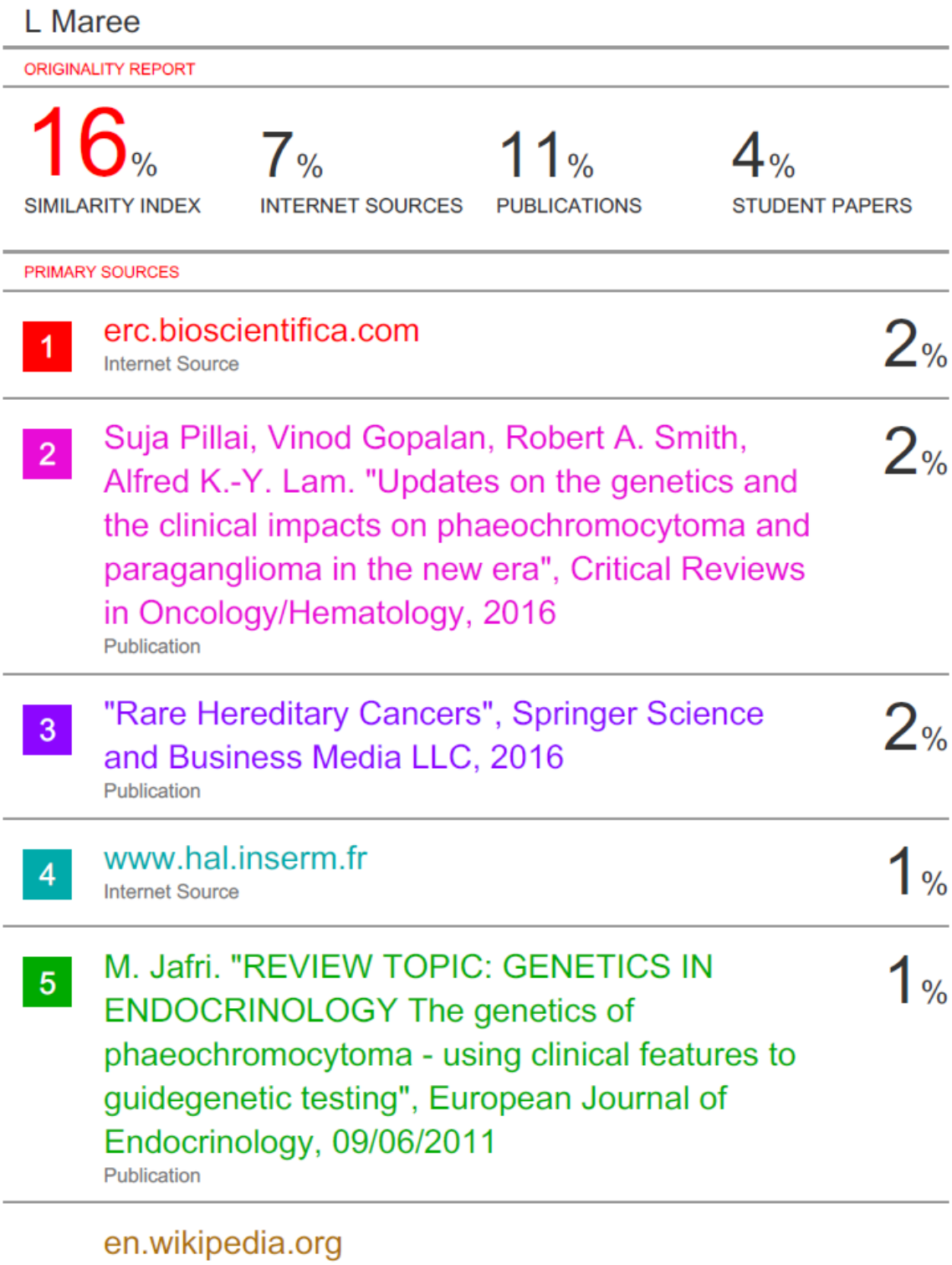

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