FLECK CORNEAL DYSTROPHY IN A SOUTH AFRICAN FAMILY

A CASE SERIES

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DEPARTMENT OF NEUROSCIENCES

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Date 22/04/2022

Author Contribution Statement

MC – Monique Camacho

MH – Michaella Hulley

SW – Susan Williams

RH – Roland Hollhumer

MC and RH devised the project. MC and RH performed clinical examination and imaging of the participants. MC obtained written informed consent from the participants. MC and RH collected saliva and blood samples from the participants. MH performed targeted exon sequencing, variant identification and mapping. All authors discussed the results and contributed to the final manuscript. MC wrote the manuscript.

Signed:

M. Camacho

M. Hulley

Susan Williams

Roland Höllhumer

Dedication

This report is dedicated to my wonderful husband Americo.

Acknowledgements

I would like to thank my supervisors without whom this project would not have been possible.

Presentations

This research was presented at the following congresses:

1. WOC 2020 (World Ophthalmology Congress) – Virtual Poster.

2. Wits Faculty of Health Sciences Biennial Research Day – Online Presentation (October 2020).

Table of Contents

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ABSTRACT

Background

Corneal dystrophies are genetically determined non-inflammatory corneal diseases. Fleck corneal dystrophy (FCD) is a rare autosomal dominant condition caused by a mutation in the phosphoinositide kinase FYVE finger (*PIKFYVE*) gene. There are few descriptions of FCD in the literature and no known studies on the genetics of this condition in sub-Saharan Africa.

Purpose

To describe the clinical features of FCD within a black South African family and to apply targeted sequencing for variant identification in the *PIKFYVE* gene.

Patients and Methods

Clinical Examination

Ten family members from three generations were examined using slit-lamp microscopy, corneal tomography and ocular coherence tomography.

Genetic Material Collection & Data Analysis

Saliva and blood samples were collected and targeted sequencing of exon 19 of *PIKFYVE* was completed using Sanger sequencing. Variants were identified and mapped.

Results

We found flecks in six individuals, five of whom had posterior embryotoxon. Six variants on exon 19 of *PIKFYVE* were identified across all family members. These include four previously identified missense variants, rs893253 (p.Thr998Ser), rs893254 (p.Gln995Leu), rs1529979 (p.Gln1183Lys) and rs999890 (p.Ser1033Ala); and two non-synonymous variants, rs1529978 (p.Asn1188=) and rs999891 (p.Arg1038=). Rs999891 has not been reported in association with FCD before. All variants were categorised as benign and identified in both cases and controls.

Conclusion

No pathogenic mutations were identified in this family that could account for the FCD. We replicated associations reported in the literature but did not find evidence to suggest that they were responsible for the phenotype. Further research is required along with expanded sequencing of the entire gene to better characterize the association of FCD with the *PIKFYVE* gene.

Background

Corneal dystrophies are bilateral non-inflammatory corneal diseases which present with a varying degree of visual impairment. These inherited corneal diseases are classified anatomically by the location in which the abnormalities occur: epithelial, subepithelial, stromal and endothelial dystrophies (1)(2).

In 1957, Francois and Neetens described an autosomal dominant dystrophy with tiny white flecks in all layers of the stroma (2). The pattern of the flecks was noted to vary from semicircular, wreath-like to curvilinear or punctuate. In the 1960's and 1970's the disorder was termed a hereditary fleck dystrophy of the cornea.

Fleck corneal dystrophy (FCD, Online Mendelian Inheritance in Man (OMIM) #121850) is a rare and non-progressive autosomal dominant condition with variable expression (2). The autosomal dominant pattern of inheritance for FCD has long been established (3)(4)(5), however displaying variable expression (6). A genetic linkage analysis on four unrelated families identified a disease locus on chromosome 2q35 (7). Within this area lies *PIKFYVE* (Gene ID: 200576) which is located at 2q34 and one of the most studied genes for FCD. Multiple mutations have been reported in *PIKFYVE* in individuals and families with FCD (8), (9).

The protein encoded by *PIKFYVE* belongs to the phosphoinositide 3-kinase family and regulates the sorting and movement of peripheral endosomes, which contain lysosomally directed fluid phase cargo, by controlling the morphogenesis and task of multivesicular bodies (12). The *PIKFYVE* gene spans more than 89kbp, contains 41 coding exons and encodes a 2,098 amino acid protein (9). Mutations in the *PIKFYVE* gene result in dilated keratocytes containing intracytoplasmic vesicles filled with complex lipids and glycosaminoglycans. Clinically, punctate stromal opacities are noted which generally present in early childhood, as young as two years but occasionally at birth (7). The tiny dot-like white flecks present throughout the corneal stroma are largely asymptomatic with normal vision but some patients may complain of photophobia (10). The stroma located between the flecks is clear and the epithelium, Bowman's layer, Descemet's membrane and the endothelium of the cornea are normal (8). The incidence of this condition remains unknown, probably due to its subtle presentation.

The aim of this study is to describe the clinical findings and the genetics of FCD in a black South African family.

Patients and Methods

This case series was performed with the approval of the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa (M2008125). The study adhered to the tenets of the Declaration of Helsinki and followed good clinical practices. All patients provided written informed consent for their medical information to be included in study analyses.

A 19-year old black male patient of Xhosa ancestry was referred to our eye clinic in Soweto, South Africa with poor vision secondary to advanced keratoconus. An incidental finding of bilateral corneal white flecks was noted. The patient's mother was subsequently examined and similar lesions were noted. A clinical diagnosis of FCD was made and another eight family members from three different generations were evaluated.

Clinical Examination

We performed thorough ophthalmological examinations and investigations on the participants which included a slit-lamp examination, autorefraction, ocular coherence tomography (OCT), corneal tomography (Oculus Pentacam®) and anterior segment photography.

Genetic Material Collection and Genetic Data Analysis

Saliva samples were collected from eight of the participants using the Oragene DNA Collection kit (DNA Genotek). Blood samples were drawn from the remaining two family members into ethylenediamine tetra-acetic acid (EDTA)-coated vacutainers. DNA was extracted from the saliva samples using the prepIT® L2P manual purification method (DNA Genotek). The DNA was then analysed using a NanoDrop Spectrophotometer, ranging in concentration from 331.4 ng/µL to 3640 ng/µL, with 260/280 ratios between 1.7 and 2. DNA was collected from the blood samples using the DNA Isolation Kit for Mammalian Blood (Roche Life Science), with concentrations of approximately 20 ng/µL and 260/280 ratios between 1.8 and 2.

The targeted exon sequencing was completed using Sanger sequencing. Exon 19 of *PIKFYVE* was sequenced using primers designed by Li *et al*. (10) as outlined in table 1. Samples were sequenced on the Applied Biosystems 3130xl Genetic Analyzer sequencer, using default settings, and the BigDye™ Terminator v3.1 Cycle Sequencing Kit.

Table 1. Primers used to sequence Exon 19 of PIKFYVE

Primer names	Forward sequence (5' – 3')	Reverse sequence (5' – 3')
AF1 and SR1	TGTACTTCTGGATTGCCACCT	CATCACAGGGCAGAGACTCA
SF1 and AR1	GGGCTGTCCAAGAGCAGTA	CAGTGTCATCCTGTAGAGGGTCT
AF2 and SR2	ACTCCCTGTGGATGACCAAC	TCCCTGAGCAGCTGTTTCTT
SF2 and AR2	TTGCAGAGCAGGTTTACTGG	TGCCTTTGAGGAGTCATTCA

Files obtained from the sequencer were used as the input for the program, Geneious 11.1.4 (http://www.geneious.com (11). This program allowed for the alignment of the sequence results to the reference sequence extracted from NCBI (NC_000002.12). Variants were identified from this alignment and mapped using the variant mapper within NCBI (https://www.ncbi.nlm.nih.gov/variation/view/?q=200576%5Bgeneid%5D). The variants were then classified as benign, likely pathogenic or pathogenic according to the ACMG/AMP guidelines (12).

Results

Clinical Findings

White flecks within the corneal stroma were found in six of the family members (Table 2). The flecks varied from subtle to prominent. Of note, posterior embryotoxon was present in five of the individuals with flecks. The proband had keratoconus but this was not found to be the case in any of the other individuals. The visual acuity of the family members was largely normal except for the proband (keratoconus), the proband's mother who is a high myope and the proband's maternal grandmother who has significant age-related lens opacities. Lens

opacities were not found in any of the other family members. All family members, except for the proband and the child too young to cooperate with examinations, exhibited normal corneal tomography (Oculus Pentacam®) and posterior segment findings. Flecks were barely visible on anterior segment OCT examination of affected individuals.

Table 2. Clinical findings and investigations

Participant	**Relationship to proband**	**Age**	**VA (Unaided) Decimal**		**Anterior Segment / Cornea**	
			OD	**OS**	**OD**	**OS**
1	Mother	41	0.4	NLP	Flecks	Flecks
2	Grandmother	64	0.25	0.5	Flecks	Flecks
3	Proband	19	0.25	0.16	Keratoconus, vernal keratoconjunctivitis, flecks	Keratoconus, vernal keratoconjunctivitis, flecks
4	Cousin	14	1.0	1.0	Subtle flecks, posterior embryotoxon	Subtle flecks, posterior embryotoxon
5	Cousin	3	-	-	Prominent corneal nerves, posterior embryotoxon	Prominent corneal nerves, posterior embryotoxon
6	Cousin	10	1.0	1.0	Clear	Scar (inferior)
7	Aunt	43	1.0	1.0	Flecks (deep and sparse), posterior embryotoxon	Flecks (deep and sparse), posterior embryotoxon
8	Cousin	18	1.0	1.25	Prominent nerves, posterior embryotoxon	Prominent nerves, posterior embryotoxon
9	Aunt	30	1.25	1.0	Clear	Clear
10	Uncle	23	1.25	1.25	Prominent nerves, flecks, posterior embryotoxon	Prominent nerves, flecks, posterior embryotoxon

Genetics

Six variants on exon 19 of *PIKFYVE* were identified across all family members (Figure 1). These include four missense variants, rs893253 (p.Thr998Ser), rs893254 (p.Gln995Leu), rs1529979 (p.Gln1183Lys) and rs999890 (p.Ser1033Ala); and two non-synonymous variants, rs1529978 (p.Asn1188=) and rs999891 (p.Arg1038=). Five of these variants have previously been described for FCD. Rs999891 has not been reported on in the literature. All variants were categorised as benign and identified in both cases and controls.

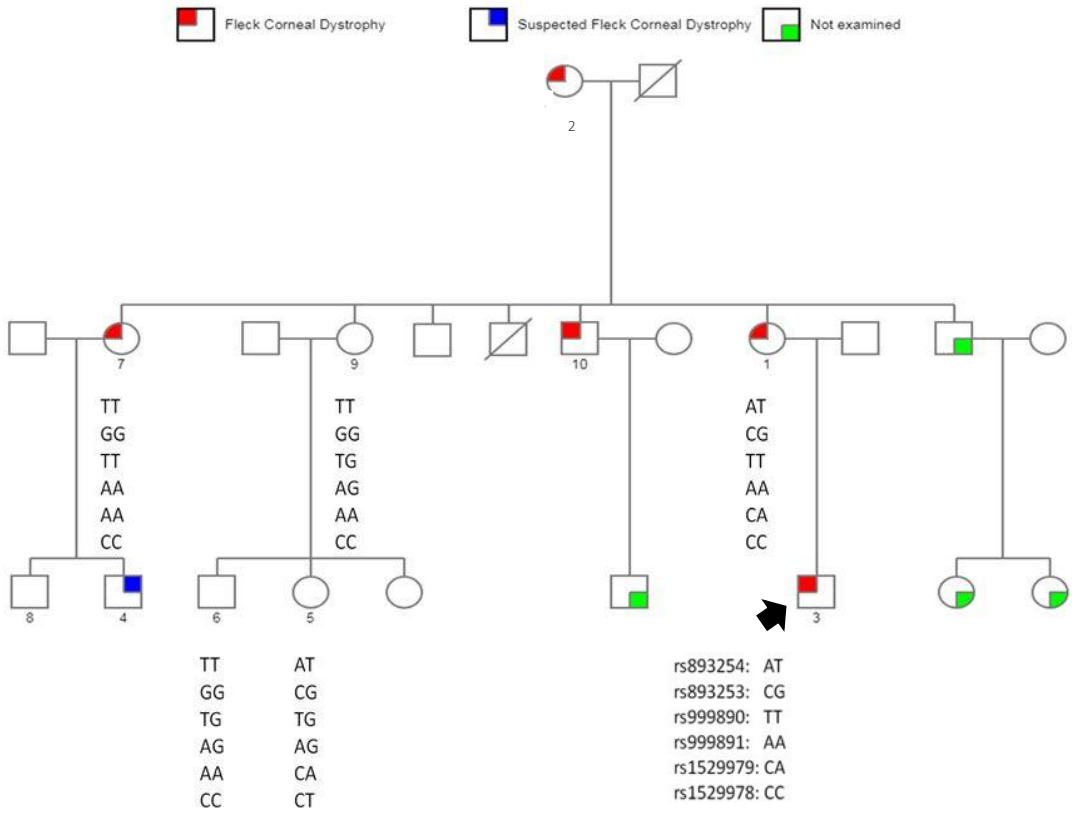

Figure 1. This 3-generation pedigree of the participants clearly illustrates an autosomal dominant pattern of inheritance. Six variants on exon 19 of PIKFYVE were identified across all family members. These include four missense variants, rs893253 (p.Thr998Ser), rs893254 (p.Gln995Leu), rs1529979 (p.Gln1183Lys) and rs999890 (p.Ser1033Ala); and two non-synonymous variants, rs1529978 (p.Asn1188=) and rs999891 (p.Arg1038=). Proband indicated with arrow.

A number of online bioinformatics tools provide central access points for protein information including function and classification. UniProt, a freely accessible online database, categorises both rs999890 and rs1529979 as pathogenic and associated with Fleck corneal dystrophy.

However, rs1529979 is categorized as benign, tolerated and neutral by the prediction tools and is observed in both affected and non-affected family members. Eight prediction tools, BayesDel_addAF, DEOGEN2, EIGEN, LIST-S2, MutationTaster, PrimateAI, REVEL and SIFT classify rs999890 as benign, tolerated or neutral, while two, FATHMM-MKL and MutationAssessor provide a damaging or pathogenic classification (see Table 3). In this study, the minor allele is only observed in non-affected family members. All six variants identified in this study were classified as benign according to the ACMG/AMP guidelines and identified in both cases and controls (12). It is important to note that there was some discrepancy between the prediction status determined by different tools.

Table 3. Variants associated with Fleck Corneal Dystrophy and identified across the family members. SIFt and Polyphen results as well as their clinical significance are noted.

Sample	Variant	Genotype	Ancestral	MAF	Variant type	Chromosome	Position	HGVS Coding	SIFT	POLYPHEN	Clinvar
1	rs893253	CG	G	C (0.09; 0.29 - ESN)	SNV (missense) - 3 prime UTR variant NMD transcript variant	2	209190528	c.2993C>G	1 (tolerated)	0 (benign)	Benign
2		CG									
3		CC									
4		GG									
5		CG									
6		GG									
7		GG									
8		GG									
9		GG									
10		CG									
1	rs893254	TA	A	A (0.09; 0.29 - ESN)	SNV (missense) - 3 prime UTR variant NMD transcript variant	2	209190519	c.2984A>T	0.22 (tolerated)	0 (benign)	Benign
2		TA									
3		AA									
4		TT									
5		TA									
6		TT									
7		TT									
8		TT									
9		TT									
10		AT									
1		TT	T			2					

2		TT									
3		TT									
4		TT									
5	rs999890	TG		G (0.10; 0.19 - ACB)	SNV (missense)		209190632	c.3097T>G	0.8 (tolerated)	0.003 (benign)	Benign
6		TG									
7		TT									
8		TT									
9		TG									
10		TT									
1	rs999891	AA	A	G (0.10; 0.19 - ACB)	SNV (synonymous) - 3 prime UTR variant NMD transcript variant	2	209190649	c.3114A>G			Benign
2		AA									
3		AA									
4		AA									
5		AG									
6		AG									
7		AA									
8		AA									
9		AG									
10		AA									
1	rs1529978	CC	C	T (<0.01; 0.03 - YRI)	SNV (synonymous)	2	209191099	c.3564T>C			Benign
2		CC									
3		CC									
4		CC									
5		CT									
6		CC									
7		CC									
8		CC									
9		CC									
10		CC									
1	rs1529979	AC	A	C (0.09; 0.29 - ESN)	SNV (missense)	2	209191082	c.3547C>A	1 (tolerated)	0 (benign)	Benign
2		AC									
3		CC									
4		AA									
5		AC									
6		AA									
7		AA									
8		AA									
9		AA									
10		AC									

MAF: Minor Allele Frequency; HGVS Coding: international standard nomenclature used to report and exchange information regarding variants found in DNA, RNA and protein sequences; SIFt: algorithm to help bridge the gap between mutations and phenotypic variations by predicting whether an amino acid substitution is deleterious; Polyphen: automatic tool for prediction

of the possible impact of an amino acid substitution on the structure and function of a human protein; SNV: single nucleotide variant; NMD: nonsense-mediated mRNA decay.

Discussion

The first multigenerational account of persons affected by FCD was described in 1977 with a detailed description of histological, biochemical and ultrastructural findings (13). This case report was also the first known account of the dystrophy in a person of African ancestry. An incidental finding of stromal flecks was noted which led to the examination of over thirty of his family members across four generations. Results of biomicroscopy, histochemistry and electron microscopy were recorded and Francois-Neetens dystrophy was diagnosed. In the same year, Goldberg et al. described variable phenotypic expressivity (5).

Advances in the analysis of FCD has allowed for enhanced understanding of the genetic and phenotypic variability of this condition. Following François and Neetens' initial description of FCD in the late 1950's, twelve mutations have since been described (9). All mutations thus far described have been heterozygous (Table 4) (9) (14).

Table 4. Summary of genetic mutations identified in Fleck Corneal Dystrophy (FCD)

Jiao *et al.* (7)	Critical region for FCD mapped to 27.9cM region of chromosome flanked by genomic markers D2S117 and D2S126.
Li *et al.* (15)	Described FCD locus in 10 unrelated European families. Mutations thought to result in truncation of PIK5K3 gene and loss of enzymatic activity.
Kawasaki *et al*. (14)	Novel c.4166_4169delAAGT mutation in Japanese woman. Mutation described as 4-base-pair deletion - may result in a framed alteration and have a profound impact on *PIKFYVE* protein function.

Gee *et al.* (9)	Described first copy number variation and the fifth frame-shift mutation associated with FCD (c.3151dupA)

The afore-mentioned descriptions of FCD represent the only publications on this topic and to our knowledge no studies on the genetics of FCD in the sub-Saharan region exist.

This study presents the sequencing results of exon 19 in the *PIKFYVE* gene. These results are described for the first time in a family from sub-Saharan Africa. Four variants previously described in the literature were found (rs893253, rs893254, rs999890 and rs1529979) and one new variant was identified (rs999891). This study is limited to one family and thus a small sample size.

Although variants have previously been identified in the *PIKFYVE* gene and associated with FCD, the variants identified in this study were classified as benign, and most likely do not contribute toward FCD development within the study population. This includes both variants that were labeled as pathogenic in UniProt, as other tools classify these as tolerated or benign, and they are also present in non-affected family members. Although the new variant has been classified as benign, it may be part of a haplotype that could be associated with FCD. Future research could sequence the remaining *PIKFYVE* gene and perhaps other potential candidate genes within the 2q34 region.

Posterior embryotoxon is an abnormality of the cornea where Schwalbe's line is anteriorly displaced resulting in a thin grey-white line visible on slit-lamp biomicroscopy (16). A large series found the prevalence of this abnormality to be 8.6% although this varies between different age groups. Posterior embryotoxon has been described in association with other anterior segment pathology, genetic and systemic abnormalities and its presence necessitates further examination (16). To our knowledge, posterior embryotoxon has not been described in association with stromal corneal dystrophies. Half of the family members examined in this study were found to have posterior embryotoxon presenting a possible genetic link between the disease processes.

In this study we present the clinical characteristics and the molecular genetics findings of a 3-generation South African family with FCD. This is the first description of the condition in sub-

Saharan Africa. No pathogenic mutations were identified in this family that could account for the FCD and expanded sequencing of the gene is required.

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Competing Interests

The authors declare that there are no professional, financial or personal interests that may have inappropriately influenced them.

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Data availability

Data are available upon reasonable request from the corresponding author, MC

Disclaimer

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors

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Ethics Declaration

Ethics clearance (Protocol Number: M131125) was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg (South Africa).

Appendix

Journal: AFRICAN VISION AND EYE HEALTH

Authors guidelines

https://avehjournal.org/index.php/aveh/pages/view/submission-guidelines#part_1

Original Research Article full structure

Title: *The article's full title should contain a maximum of 95 characters (including spaces).*

Abstract: *The abstract, written in English, should be no longer than 250 words and must be written in the past tense. The abstract should give a succinct account of the objectives, methods, results and significance of the matter. The structured abstract for an Original Research article should consist of six paragraphs labelled Background, Aim, Setting, Methods, Results and Conclusion.*

- *Background: Summarise the social value (importance, relevance) and scientific value (knowledge gap) that your study addresses.*
- *Aim: State the overall aim of the study.*
- *Setting: State the setting for the study.*
- *Methods: Clearly express the basic design of the study, and name or briefly describe the methods used without going into excessive detail.*
- *Results: State the main findings.*
- *Conclusion: State your conclusion and any key implications or recommendations.*

Do not cite references and do not use abbreviations excessively in the abstract.

Introduction: *The introduction must contain your argument for the social and scientific value of the study, as well as the aim and objectives:*

- *Social value: The first part of the introduction should make a clear and logical argument for the importance or relevance of the study. Your argument should be supported by use of evidence from the literature.*
- *Scientific value: The second part of the introduction should make a clear and logical argument for the originality of the study. This should include a summary of what is already known about the research question or specific topic, and should clarify the*

knowledge gap that this study will address. Your argument should be supported by use of evidence from the literature.

- *Conceptual framework: In some research articles it will also be important to describe the underlying theoretical basis for the research and how these theories are linked together in a conceptual framework. The theoretical evidence used to construct the conceptual framework should be referenced from the literature.*
- *Aim and objectives: The introduction should conclude with a clear summary of the aim and objectives of this study.*

Research methods and design: *This must address the following:*

- *Study design: An outline of the type of study design.*
- *Setting: A description of the setting for the study; for example, the type of community from which the participants came or the nature of the health system and services in which the study is conducted.*
- *Study population and sampling strategy: Describe the study population and any inclusion or exclusion criteria. Describe the intended sample size and your sample size calculation or justification. Describe the sampling strategy used. Describe in practical terms how this was implemented.*
- *Intervention (if appropriate): If there were intervention and comparison groups, describe the intervention in detail and what happened to the comparison groups.*
- *Data collection: Define the data collection tools that were used and their validity. Describe in practical terms how data were collected and any key issues involved, e.g. language barriers.*
- *Data analysis: Describe how data were captured, checked and cleaned. Describe the analysis process, for example, the statistical tests used orsteps followed in qualitative data analysis.*
- *Ethical considerations: Approval must have been obtained for all studies from the author's institution or other relevant ethics committee and the institution's name and permit numbers should be stated here.*

Results*: Present the results of your study in a logical sequence that addresses the aim and objectives of your study. Use tables and figures as required to present your findings. Use quotations as required to establish your interpretation of qualitative data. All units should conform to the SI convention and be abbreviated accordingly. Metric units and their international symbols are used throughout, as is the decimal point (not the decimal comma).*

Discussion*: The discussion section should address the following four elements:*

- *Key findings: Summarise the key findings without reiterating details of the results.*
- *Discussion of key findings: Explain how the key findings relate to previous research or to existing knowledge, practice or policy.*

- *Strengths and limitations: Describe the strengths and limitations of your methods and what the reader should take into account when interpreting your results.*
- *Implications or recommendations: State the implications of your study or recommendations for future research (questions that remain unanswered), policy or practice. Make sure that the recommendations flow directly from your findings.*

Conclusion: *Provide a brief conclusion that summarises the results and their meaning or significance in relation to each objective of the study.*

Acknowledgements: *Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution. Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named. Refer to the acknowledgement structure guide on our Formatting Requirements page.*

Also provide the following, each under their own heading:

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Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Dr MdeG Camacho
School of Clinical Medicine
Department of Neurosciences
Division of Opthalmology
Medical School
University

E-mail: moniquecamacho@live.co.za

CC: Supervisor: Drs R Hollhumer & S Williams; Ms M Hulley
<hollhumer@gmail.com>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2020/09/22

REF: *R14/49*

PROTOCOL NO: **M2008125** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Fleck corneal dystrophy in a Black South African family - a retrospective case series*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PROTOCOL ASSESSORS MEETING

Candidate Full Name: **DR CAMACHO MONIQUE**

Student Number: **0303881P** Date: **19 FEBRUARY 2020**

School / Department / Division: **Clinical Medicine/Department of Neurosciences/Division of Ophthalmology**

1. **Type of study** *(tick all that apply)*:

- o Quantitative
- o Qualitative
- o Mixed Methods
- ◂ Laboratory
- ● Clinical
- o Other, please specify.....

2. **Is title of the study appropriate** (*preferably fewer than 20 words*)? Yes ☒ No ☐

Comments:

3. **Are the study objectives clear and linked to the research aim and title?** Yes ☒ No ☐

Comments: In objective — comparison to literature should be a discussion rather than objective.

4. **Is the design of the study appropriate to meet the study objectives?** Yes ☒ No ☐

Comments:

11 March 2019/MP

1

5. **Are the proposed methods and tools appropriate to meet the research objectives?** [X] Yes [ ] No

Comments: Document pedigree

6. **Is the study feasible within the resources of:**

a) The applicant? [X] Yes [ ] No

b) The department? [X] Yes [ ] No

c) The time frame? [X] Yes [ ] No

7. **If this is a PhD protocol assessment:**

a) Is the content original? [ ] Yes [ ] No

b) Does the content show the scope and depth of a PhD? [ ] Yes [ ] No

Comments:

Do you recommend:

i. Additional revision/amendment of the protocol? Please be specific on the recommendations being made:

ii. The appointment of the proposed Supervisor? [ ] Yes [X] No

Nominee/s:

11 March 2019/MP

iii. The appointment of the proposed Co-Supervisor/s and/or additional co-supervisors? Yes | No

Nominee/s: _____

_____

_____

iv. Has the Chair of the Assessor Group signed the RECOMMENDATION FOR APPOINTMENT OF SUPERVISOR(S) OF RESEARCH REPORT, DISSERTATION OR THESIS form? Please attach. Yes | No

v. Has the Chair informed the student and supervisor about the Wits ethics requirements, and that if required, they must have either a Wits Human Research Ethics clearance certificate or a Wits Animal Research Ethics clearance certificate? Yes | No

vi. Based on the protocol provided (including any proposed changes by the protocol assessor group), does the student require:

1.	Human Research Ethics clearance certificate	Yes / No
2.	Animal Research Ethics clearance certificate	Yes / No
3.	No human or animal ethics certificate is required	Yes / No
4.	Unclear, will seek appropriate guidance from the HREC/AREC committees	Yes / No

vii. Has the Postgraduate student and supervisor/s signed the ethics declaration form Yes | No

Overall recommendation regarding the protocol:

i. Revision of the protocol to the satisfaction of the Supervisor (**NB: if HoD approval is also required, please specify**): Yes | No
*(**Candidate**: one copy, list of corrections with page numbers and Supervisor approval letter – submit to PG Office).*

ii. Revision of the protocol to the satisfaction of the Assessor Group/Chair: Yes | No
*(**Candidate**: one copy, list of corrections with page numbers, Supervisor approval letter – submit to PG Office and PG Office to forward to the Assessor Group Chair).*

iii. Revision of the protocol and resubmission of the revised protocol to the next Assessor Group Meeting: Yes | No
*(**Candidate**: six copies, list of corrections with page numbers, Supervisor approval letter – submit one copy to PG Office / 5 to school assessor group administrator / for PhD, all six copies to be submitted to the PG Office).*

iv. Candidate goes ahead (*no revision required*): Yes | No

11 March 2019/MP

3

Details of Assessors:

Name:	Email:	Sign:
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Details of Assessor Group Chair:

Name:	Email:	Sign:
PROFESSOR A MOCHAN	Andre.mochan@wits.ac.za	

Date: **19 FEBRUARY 2020**

PROTOCOL

Fleck Corneal Dystrophy in a black South African family

A retrospective case series

Dr Monique De Gouveia Camacho
BSc Hons (Wits) MBBCh (Wits)

Student Number: 0303881P

Registrar

Department of Neurosciences. Division: Ophthalmology
University of Witwatersrand

Supervisors

Dr Roland Höllhumer, Consultant

Division of Ophthalmology, University of the Witwatersrand

Miss Michaella Hulley, PhD candidate

Division of Human Genetics - School of Pathology, University of Witwatersrand

Dr Susan Williams, Senior Consultant

Division of Ophthalmology, University of the Witwatersrand

Contents

Fleck Corneal Dystrophy in a black South African family

A case series

Introduction and Literature Review

. Background

1. The Corneal Dystrophies: History and Classification

Corneal dystrophies are bilateral non-inflammatory corneal diseases (1). In 1890, Arthur Groenouw described two patients with "noduli corneae," with one of the patients having granular corneal dystrophy and the other, macular corneal dystrophy (2). Although corneal examination was limited before the slit lamp became a commonly used piece of equipment, the two diseases were termed corneal dystrophies by Groenouw. Fuchs then went on to describe a group of eye diseases with dystrophic changes. These dystrophic tissues were a result of a paucity of necessary nutrients, hormones, blood, and nerve supply(3).

Clinically, the corneal dystrophies are classified into the following groups on the basis of the anatomical location of the abnormalities: epithelial and subepithelial dystrophies, epithelial-stromal TGFBI (transforming growth factor beta induced) dystrophies, stromal dystrophies, and endothelial dystrophies (2). Most corneal dystrophies affect visual acuity to varying degrees and do not present with systemic problems. Dystrophies may be inherited in an autosomal dominant, autosomal recessive or X-linked recessive fashion. Recent genetic studies on corneal dystrophies have yielded molecular level insight into some of these disorders. This novel knowledge has lead to changes in the classification of the corneal dystrophies and to the development of new therapeutic approaches.

2. Fleck Corneal Dystrophy: Early identification, genetics and clinical features

In 1957, Francois and Neetens described an autosomal dominant dystrophy with tiny white flecks in all layers of the stroma (4). The pattern of the flecks were noted to vary from semicircular, wreath-like to curvilinear or punctuate. In the 1960's and 1970's the disorder was termed a hereditary fleck dystrophy of the cornea. The first multigenerational account of affected persons was described in 1977 with a detailed description of histological, biochemical and ultrastructural findings (5). This case report was also the first known account of the dystrophy in a person of African ancestry. The proband was a diabetic black man who had lost his vision following retinal and ciliary artery occlusions as a result of phycomycosis. An incidental finding of stromal flecks was noted which led to the examination of over thirty of his family members across four generations. Results of biomicroscopy, histochemistry and electron microscopy were recorded and Francois-Neetens dystrophy was diagnosed. In the same year, Goldberg *et al.* described variable phenotypic expressivity (6).

Fleck corneal dystrophy (FCD, Online Mendelian Inheritance in Man (OMIM) #121850) is a rare and non-progressive autosomal dominant condition with variable expression (4). The autosomal dominant pattern of inheritance for FCD has long been established (7); (8)and(6), however displaying variable expression (9). A genetic linkage analysis on four unrelated families identified a disease locus on chromosome 2q35 (10). Within this vicinity lies *PIKFYVE* (Gene ID: 200576), located at 2q34, one of the most studied genes for FCD. Multiple mutations have been reported in *PIKFYVE* in individuals and families with FCD(11), (12).

The protein encoded by *PIKFYVE* belongs to the phosphoinositide 3-kinase family and regulates the sorting and movement of peripheral endosomes which contain lysosomally directed fluid phase cargo, by controlling the morphogenesis and task of multivesicular bodies (12). The *PIKFYVE* gene spans more than 89kbp, contains forty one coding exons and encodes a 2,098 amino acid protein (12).

Mutations in the *PIKFYVE* gene result in dilated keratocytes containing intracytoplasmic vesicles filled with complex lipids and glycosaminoglycans. Clinically, punctate stromal opacities are noted which generally present in early childhood, as young as two years but occasionally at birth (10). The tiny dot-like white flecks present throughout the corneal stroma are largely asymptomatic with normal vision but some patients may complain of photophobia. The stroma located between the flecks is clear and the epithelium, Bowman's layer, Descemet's membrane and the endothelium of the cornea are normal. The incidence of this condition remains unknown, probably due to its subtle presentation.

3. Descriptions of *PIKFYVE* gene mutations in the literature

Advances in the analysis of FCD has allowed for enhanced understanding of the genetic and phenotypic variability of this condition. Following François and Neetens' initial description of FCD in the late 1950's, twelve mutations have since been described (12). All mutations thus far described have been heterozygous.

Table 1: Summary of genetic mutations in the literature

Li *et al* (14)	Described FCD locus in 10 unrelated European families. Mutations thought to result in truncation of PIK5K3 gene and loss of enzymatic activity.
Kawasaki *et al* (3)	Novel c.4166_4169delAAGT mutation in Japanese woman. Mutation described as 4-base-pair deletion - may result in a framed alteration and have a profound impact on *PIKFYVE* protein function.
Gee *et al* (12)	Described first copy number variation and the fifth frame-shift mutation associated with FCD (c.3151dupA)

The afore-mentioned descriptions of FCD represent the only publications on this topic and to my knowledge no studies on the genetics of FCD in the sub-Saharan region exist.

Study Aim

The aim of this study is to describe FCD in a black South African family. This description is thought to be the first of its kind in this population group.

Study Objectives

- To describe FCD in a black South African family
    - Clinical findings will be described and compared to those in current literature
    - Functional and structural eye changes will be described using special investigations. These investigations include autorefraction, ocular coherence tomography (OCT), anterior segment photography and corneal topography
- To describe the results of genetic analysis of the *PIKFYVE* gene in this family.
    - Identification of genes previously described in literature
    - To identify haplotypes and report on their significance

Study Design

Record review

The records and clinical investigations of ten family members will be examined to identify clinical features of FCD. The family in question was initially identified at the St John Eye Hospital in Soweto. The family was enrolled in a gene identification study in corneal dystrophy study in December 2017 (See Appendix 1). The principal investigator of the study has been approached and has given permission for the use of all data in this regard.

Genetic Analysis

The initial genetic analysis of the saliva and blood samples took place at the Division of Human Genetics, Department of Pathology at the NHLS in Braamfontein.

Study procedure

I performed the initial clinical examinations and genetic material collections of the family members with the assistance of Dr Roland Höllhumer, my main MMed supervisor. The procedures used are outlined below.

Clinical Examinations

The participants of the original study all had thorough ophthalmological examinations and investigations/imaging which included:

- **Autorefraction**
  - A tool used during an eye exam to provide an objective measurement of a person's refractive error and prescription for glasses

- **OCT (Ocular Coherence Tomography)**
  - A medical imaging technique which captures micro-resolution 2D and 3D images of biological tissue

- **Corneal topography (Oculus Pentacam ®)**
  - A non-invasive medical imaging technique for mapping the surface of the cornea

- **Anterior segment photography**
  - Low-tech imaging of the anterior structures of the eye

Genetic Material Collection and Genetic Data Analysis

Saliva samples were collected from eight of the participants using the Oragene DNA Collection kit (DNA Genotek), while blood samples were drawn from the remaining two family members into ethylenediamine tetra-acetic acid (EDTA)-coated vacutainers. DNA was extracted from the saliva samples using the prepIT® L2P manual purification method (DNA Genotek). The DNA was then analysed using a NanoDrop Spectrophotometer, ranging in concentration from 331.4 ng/µL to 3640 ng/µL, with 260/280 ratios between 1.7 and 2. DNA was collected from the blood samples using the DNA Isolation Kit for Mammalian Blood (Roche Life Science), with concentrations of approximately 20 ng/µL and 260/280 ratios between 1.8 and 2.

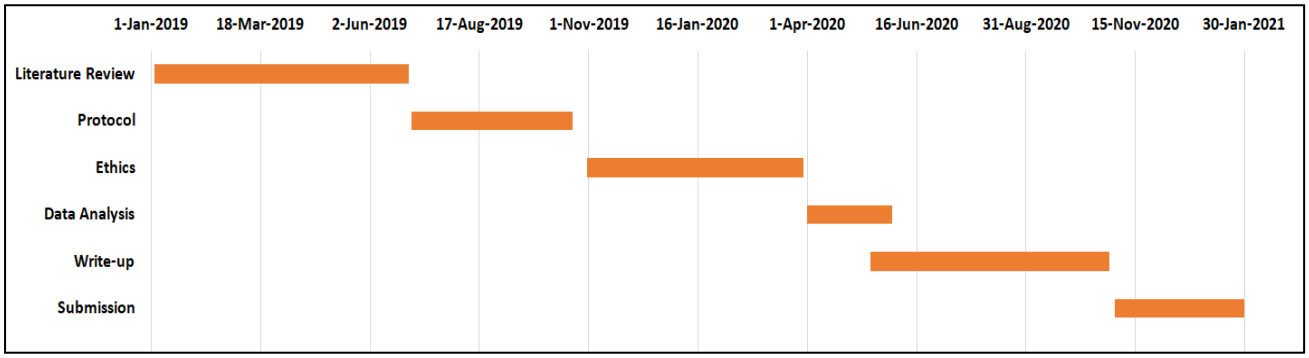
The targeted exon sequencing was completed using Sanger sequencing by Miss Michaella Hulley. Exon 19 of *PIKFYVE* was sequenced using primers designed by Li *et al*. (2005). Samples were sequenced on the Applied Biosystems 3130xl Genetic Analyzer sequencer, using default settings, and the BigDye™ Terminator v3.1 Cycle Sequencing Kit.

Files obtained from the sequencer were used as the input for the program, Geneious 11.1.4 (http://www.geneious.com, Kearse et al., 2012). This program allowed for the alignment of the sequence results to the reference sequence extracted from NCBI (NC_000002.12). Variants were identified from this alignment and mapped using the Variant mapper within NCBI (https://www.ncbi.nlm.nih.gov/variation/view/?q=200576%5Bgeneid%5D).

The pathogenicity index for the identified mutations were calculated *in silico* using Sorting Intolerant from Tolerant (SIFT) (15), and Polymorphism Phenotyping V2 (Poly-phen-2) (15). The frequencies of the variants in African populations were determined using the Population Genetics tool in Ensembl (https://www.ensembl.org/index.html.

I will be analyzing the data collected in the corneal dystrophy study and reporting on the findings as per my study objectives.

Predicted Timeline



Ethics

An ethics application for the use of clinical records for this retrospective review will be submitted.

I have requested permission for the use of the records from Dr Nadia Carstens. Dr Carstens is the principal investigator of a corneal dystrophy study for which ethics clearance was previously obtained: Human Research Ethics Committee (Medical) of the University of the Witwatersrand, South Africa (Protocol number: M131125). See appendix A

Funding

I will be covering all printing/ink and paper costs.

Possible limitations

As this is a retrospective data analysis, there may be some missing clinical or genetic data.

Reporting plans

- Write up as an MMED research report.
- Publish as an article in a peer-reviewed scientific journal.
- Presentation of study findings at the Ophthalmological Society of South Africa (OSSA) congress.

Authorship and contributions

The study was conducted by Dr M. Camacho, the principal researcher and registrar in the Department of Ophthalmology, University of the Witwatersrand. Dr Roland Höllhumer provided assistance with the identification and examination of participants. Miss Michaella Hulley was responsible for the DNA isolation and targeted Sanger sequencing. Dr Susan Williams provided research advice. Dr Nadia Carstens is the original principal investigator of the corneal dystrophy study.

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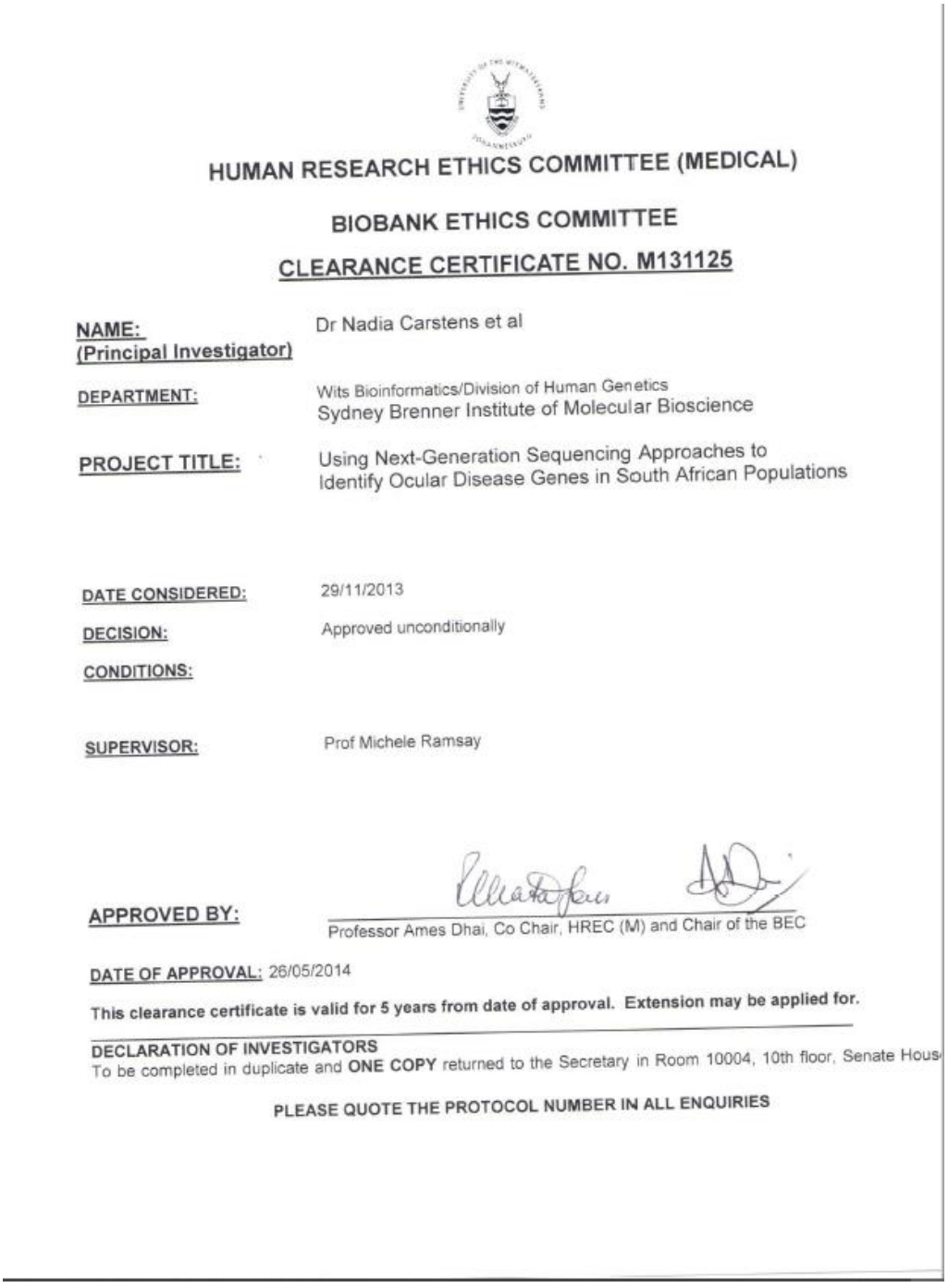
APPENDIX A



HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

BIOBANK ETHICS COMMITTEE

CLEARANCE CERTIFICATE NO. M131125

NAME: (Principal Investigator)	Dr Nadia Carstens et al
DEPARTMENT:	Wits Bioinformatics/Division of Human Genetics Sydney Brenner Institute of Molecular Bioscience
PROJECT TITLE:	Using Next-Generation Sequencing Approaches to Identify Ocular Disease Genes in South African Populations
DATE CONSIDERED:	29/11/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Michele Ramsay

APPROVED BY: Professor Ames Dhai, Co Chair, HREC (M) and Chair of the BEC

DATE OF APPROVAL: 26/05/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS
To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate Hous

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