Exome Sequencing of individuals with vitiligo and their relatives with and without autoimmune disorders

Kentie Rofhiwa Rabinda

A research report (in the format of a "submissible" paper) submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Genomic Medicine).

Johannesburg, 2023

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09 December 2022

Dear examiner,

Research Report submitted to the faculty of Health Sciences by Ms. Kentie Rofhiwa Rabinda in Fulfilment of the requirements for the degree of Master of Science in Medicine (Genomic Medicine).

Thank you for agreeing to examine research a research report titled "Exome Sequencing of individuals with vitiligo and their relatives with and without autoimmune disorders."

Ms. **Kentie Rofhiwa Rabinda**, a candidate for MSc in Genomic Medicine, is submitting her Research Report in a "Submissible" format. The Research Report contributes 30% towards the final mark in fulfillment of this MSc degree requirements. The Research Protocol assessed and approved by the Faculty of Health Sciences is attached below (Appendix B) for a detailed literature review but is not for examination purposes.

The candidate and her supervisor, Dr. Thandiswa Ngcungcu, have chosen to submit the Research Report to Frontiers in Immunology Journal, therefore the manuscript was written following the above-mentioned journal authors' guidelines. These guidelines have been attached for your reference (Appendix D). There are deviations made from this journal i.e. The tables and figures are placed in the text and not submitted separately as per the Frontiers guidelines, for ease of reading. Additionally, line numbers are not inserted and a line spacing of 1,5 was used instead of a line spacing of 1 for readability. Supporting documents are called Appendices throughout the manuscript ratherthan supplementary documents.

Please refer to the Table of Contents for a complete list of the contents provided in this research report.

Yours sincerely,

Student: Kentie Rabinda **Supervisor: Thandiswa Ngcungcu**

Declaration

I, Kentie Rabinda, declare that this research report (in the format of a "submissible" paper) is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine (Genomic Medicine) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of the candidate)

06 June 2023 at Johannesburg

Contribution of the candidate to the paper

Declaration: Student's contribution to the article and agreement of co-author.

I, Kentie Rabinda, student number, 1132300 declare that this Research Report is my own work and that I contributed significantly towards the research findings presented in the paper intended for publication below.

Signature of the Student: 

Date: 05/12/2022

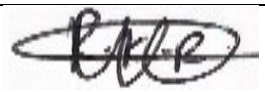
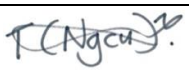
Name of Supervisor: Dr. Thandiswa Ngcungcu

Signature of Supervisor: 

Date: 07/12/2022

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of her Research Report.

Article Title: Exome Sequencing of individuals with vitiligo and their relatives with and without autoimmune disorders.

Authors	Names	Signatures	Date
1st author	Kentie Rabinda		05/12/2022
2nd author	Thandiswa Ngcungcu		07/12/2022

Dedication

This Report is dedicated to my mother, Selina Nditsheni Rabinda.

Abstract

Autoimmune disorders result from the immune system attacking and damaging its healthy cells because of an acquired immune system malfunction. Vitiligo is an example of an autoimmune disorders. It is a common depigmentation skin disorder with an estimated prevalence of 0.5–2% of the population worldwide. It is shown by selective loss of melanocytes causing non- scaly, chalky-white macules. Individuals with vitiligo often have other autoimmune disorders or first-degree relatives with at least one other autoimmune disorder. This study aimed to identify genetic variants in selected genes in individuals with vitiligo and their unaffected close relatives who have other autoimmune disorders. Upon DNA extraction, whole exome sequencing was performed, and five non- major histocompatibility complex (MHC) genes were analysed. Identified variants were annotated on Ensembl Variant Effect Predictor (VEP) and compared between individuals with vitiligo and their close relatives with and without autoimmune disorders in a family-specific manner. Eight variants were identified in two families, however, the three missense variants in the *NLRP1* gene (rs12150220, rs11651270, and rs2301582) were observed between and across two families in individuals with autoimmune disorders. *SMOC2* rs13208776, was identified as a risk locus for vitiligo in individuals from two Romanian isolate villages with vitiligo and other autoimmune disorders. None of the genotyped individuals were homozygous for the minor allele (A) and 2/12 individuals were heterozygous therefore it is unlikely that rs13208776 has any role in the development of vitiligo or any of the autoimmune disorders present in the two families. This study showed that *NLRP1* gene variants segregated with vitiligo and autoimmune disorders.

Acknowledgment

I would like to thank my supervisor for the guidance and support you have given me throughout the year. Thank you so much for not giving up on me. I'd also like to thank Dr.Lindie Lamola for guiding me through Lab work, I really appreciate it so much. Dr. Khuthala Mnika, thank you so much for being my pillar of strength in my journey. I am grateful.

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Research Report in the format of a “submissible” paper

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Keywords: Autoimmune disorders, Vitiligo, non-major histocompatibility complex(MHC) genes, Whole exome sequencing, *SMOC2*

Abstract

Autoimmune disorders result from the immune system attacking and damaging its healthy cells because of an acquired immune system malfunction. Vitiligo is an example of an autoimmune disorder. It is a common depigmentation skin disorder with an estimated prevalence of 0.5–2% of the population worldwide. It is shown by selective loss of melanocytes causing non- scaly, chalky-white macules. Individuals with vitiligo often have other autoimmune disorders or first-degree relatives with at least one other autoimmune disorder. This study aimed to identify genetic variants in selected genes in individuals with vitiligo and their unaffected close relatives who have other autoimmune disorders. Upon DNA extraction, whole exome sequencing was performed, and five non- major histocompatibility complex (MHC) genes were analysed. Identified variants were annotated on Ensembl Variant Effect Predictor (VEP) and compared between individuals with vitiligo and their close relatives with and without autoimmune disorders in a family-specific manner. Eight variants were identified in two families, however, the three missense variants in the *NLRP1* gene (rs12150220, rs11651270, and rs2301582) were observed between and across two families in individuals with autoimmune disorders. *SMOC2* rs13208776, was identified as a risk locus for vitiligo in individuals from two Romanian isolate villages with vitiligo and other autoimmune disorders. None of the genotyped individuals were homozygous for the minor allele (A) and 2/12 individuals were heterozygous therefore it is unlikely that rs13208776 has any role in the development of vitiligo or any of the autoimmune disorders present in the two families. This study showed that *NLRP1* gene variants segregated with vitiligo and autoimmune disorder.

Introduction

Autoimmune disorders (AID's) are caused by the immune system attacking and damaging its healthy cells because of an acquired immune system malfunction (Frisoli et al., 2020). AID's are caused by a complex interaction of genetic and environmental factors (Narita et al., 2011). They are usually identified by the presence of antibodies in the body, which attack specific self-cells by binding to their receptors, resulting in cell lysis and, ultimately, disease pathogenesis (Iannella et al., 2016).

AID's include Hashimoto's disease, psoriasis, rheumatoid arthritis (RA), and vitiligo (Baldini et al., 2017). Co-occurrence of at least two AID's has been observed in people with an autoimmune disorder and/or their first-degree relatives (Richard-Eaglin et al., 2018). This study revealed that at least 30% of vitiligo patients have other AID's, and many studies on vitiligo patients from various populations have revealed a strong epidemiological link with several AIDs. Gopal and colleagues (2014) found individuals with vitiligo often also had diabetes mellitus and/or hypothyroidism (thyroid dysfunction) which are all AID's. This implies that common genetic factors underlie the pathogenesis of AID's, as well as a common genetic background that predisposes them to autoimmunity (Gill et al., 2016). Most AID's have a genetic cause linked to genes in the major histocompatibility complex (MHC), but non-MHC genes have also been strongly associated with AIDs susceptibility (Iannella et al., 2016, Patwardhan et al., 2013).

Vitiligo is a skin disorder caused by melanocyte loss, which results in skin depigmentation in the affected areas (Ezzedine et al., 2011, Rajendiran et al., 2020). It is the most common disorder of the skin with roughly 70 million people affected globally (Iannella et al., 2016). Vitiligo has a worldwide prevalence of approximately 0.5-2% in adults and children (Iannella et al., 2016, Gopal et al 2014) affecting all ethnic groups. Vitiligo's average age of onset is usually before 30 years of age (Silverberg et al., 2015).

Vitiligo is clinically classified into two main types: segmental vitiligo (SV) and non-segmental vitiligo (NSV)) and an additional type called unclassified vitiligo (Baldini et al., 2017). NSV accounts for a bulk of vitiligo cases and the depigmentation is bilateral, it spreads throughout the body (Ezzedine et al., 2011), whilst SV has unilateral depigmentation that does not cross the midline (Baldini et al., 2017) with earlier age of onset as compared to NSV (Ezzedine et al., 2011).

The complex interaction of genetic and environmental factors is the main cause of vitiligo (Frisoli et al., 2020). The heritability of vitiligo is 50-75% across various populations (Alkhateeb et al., 2003). It also shows a modest correlation in which patients' first-degree relatives have higher rates of vitiligo and other AID's than the general population (Alkhateeb et al., 2003). The concordance of vitiligo was

found to be 23% in identical twins (Czajkowski et al., 2014). Common genetic variants account for 70% while rare genetic variants account for 30% of the genetic risk with the development of vitiligo (Spritz, & Santorico, 2021).

Non-major histocompatibility complex (MHC) loci have been identified as risk factors among the genetic factors involved in the development of the vitiligo immune response and they may also increase the risk of various AIDs (Garcia-Melendez et al., 2014). Five non-major histocompatibility complex (non-MHC) genes were chosen and analyzed for mutations that could be the cause of vitiligo and other AIDs in two families. Thus, this study aims to identify genetic variants in selected non-major histocompatibility complex (MHC) genes of individuals with vitiligo and their unaffected close relatives who have other autoimmune disorders.

Through findings of association studies and family studies, genetic factors are known to play important roles in vitiligo (Shen et al., 2016). Genome-wide association studies (GWAS) haveidentified more than 40 robust susceptible loci associated with vitiligo (Shen et al., 2016). The SPARC Related Modular Calcium Binding 2 *(SMOC2)* gene on 6q27 wasidentified as a risk locus for vitiligo in individuals with vitiligo and other AIDs from two adjacent Romanian isolate villages (Alkhateeb et al., 2010). It encodes the SMOC2 protein which is found in the epidermis' basal layer and promotes the attachment of primary keratinocytes (Alkhateeb et al., 2010). The *SMOC2* rs13208776 variant, located in intron 4 of the *SMOC2* gene (Shen et al., 2016) was found to be associated with vitiligo in Romanians. This association, however, was not found in Jordanians (Shen et al., 2016). In Jordanian women, the rs13208776 SNP had a marginal association with Hashimoto's disease (Alkhateeb, et al., 2013). Thus, *SMOC2* rs13208776 SNP was genotyped in this study to determine if this variant is present in two South African families of Indian descent affected by vitiligo and other autoimmune disorders.

The aim of this study was to identify genetic variants in selected non-MHC genes of individuals with vitiligo and their unaffected close relatives who have other autoimmune disorders. The pedigrees for Families 1 and 2 are suggestive of a shared mutations (mutation found in both families) that are probably high impact, family-specific and show an autosomal dominant mode of inheritance with incomplete penetrance and segregate insuccessive generations. The mutation/s are likely to be pleiotropic, but the mutations could be the same in both families or be family specific.

Materials and Methods

Participants

This research project forms part of the umbrella project “The genetic aetiology of vitiligo in Africans”, approved by the University of Witwatersrand Human Research Ethics Committee (HREC, M200504).

This is a sub-study of the umbrella project and was approved by the University of the Witwatersrand Human Research Ethics Committee HREC (M200504, M220389, Appendix C).

Nine individuals of Indian descent from two families with vitiligo across multiple generations were included in the study. For Family 1 (Figure 1), six individuals were included in the study; a proband with vitiligo III-1 (VIT113) and other autoimmune disorders, her unaffected mother II-2 (VIT113E), and unaffected fraternal twin III-2 (VIT113D), her unaffected cousin III-12 (VIT113B whose father has vitiligo) along with a sister (III-6 (VIT13C)) who has psoriasis and rheumatoid arthritis (RA) and another with eczema, cafe au lait spots and unclassified skin allergies III-4 (VIT113G). A nuclear family (Family 2) with a male proband VI-1 (VIT118) with vitiligo and Hashimoto disease, his mother V-2 (VIT118B) who has Hashimoto disease, and his father V-1 (VIT118A) who has alopecia were also included. The nine individuals and an additional three individuals, III-8 (VIT113F), VIT113I, and VIT113H from Family 1 were also genotyped for the *SMOC2* rs13208776 SNP.

DNA extraction

DNA was extracted from venous blood using the salting out method (Miller et al., 1988). The GenFind V3 50 prep kit method (Seipp, et al., 2010) was also used to extract samples that had not been successfully extracted with the salting out method (III-1(VIT113A), III-6 (VIT113C). The DNA quality was assessed using gel electrophoresis (0.8% agarose gel). The quality and quantity (concentration of DNA per µl) of the DNA was assessed using the Nanodrop™2000 (ThermoFisher Scientific) to determine the concentration and A260/A280 and A260/A230 ratios.

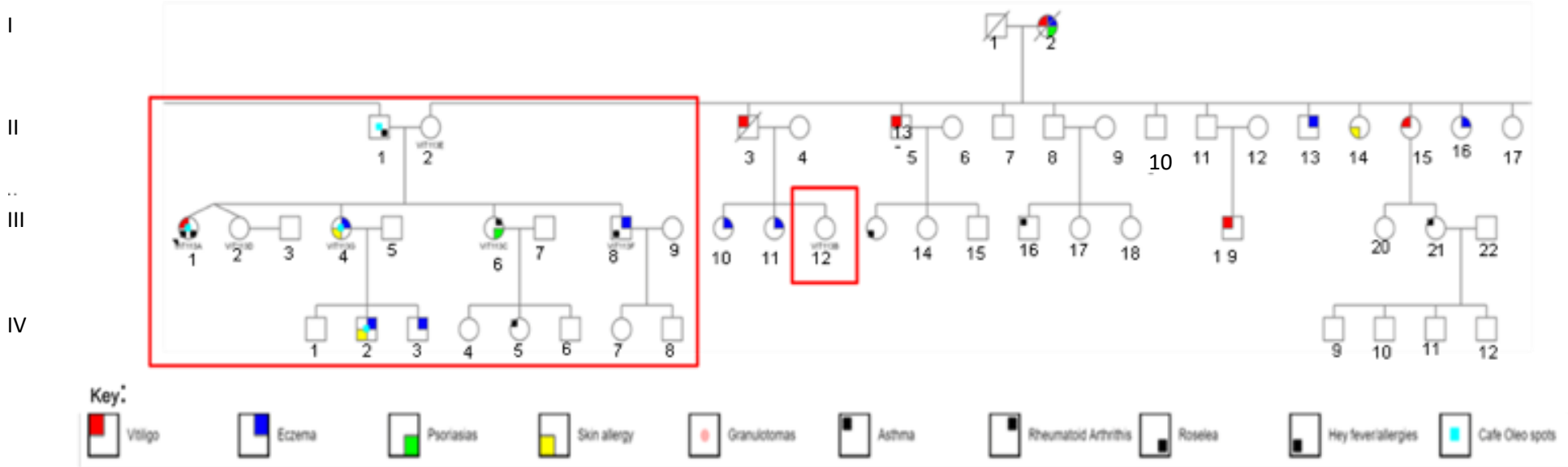


Figure 1: Pedigree of Family 1. The probands'(III-1) maternal side shows multigenerational tracking of vitiligo and various autoimmune diseases. The individuals with a VIT ID shown by the red blocks are used in this study. Individuals II-2 (VIT 113E), the proband III-1 (VIT 113A), IIII-4 (VIT 113G), III-6 (VIT 113C), III-12 (VIT113B) were used for exome sequencing, and in addition to these individuals, III-2 (VIT113D), III-8 (VIT113F) VIT113D, VIT113H and VIT113I were genotyped for the *SMOC2* rs13208776 SNP. The disorder key is indicated at the bottom of the image.

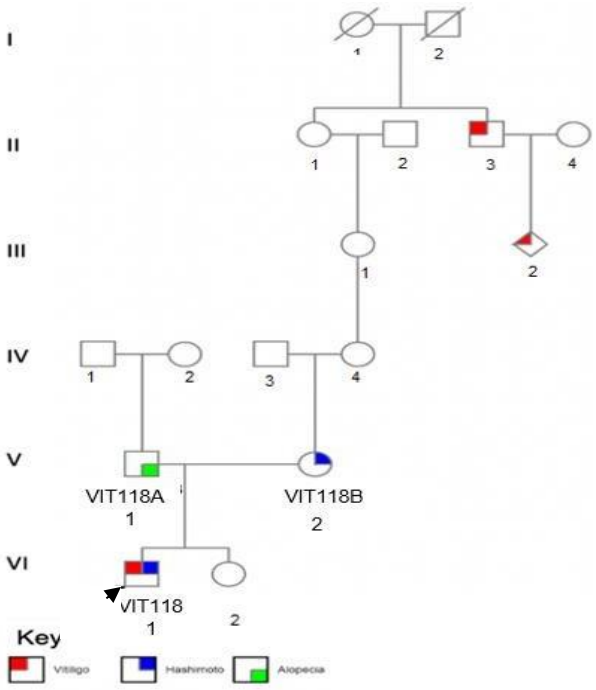


Figure 2: Pedigree of Family 2. The probands' (VI- 1) maternal side shows multigenerational tracking of vitiligo. The individuals with a VIT ID are used in this study. Individuals V-1(VIT 18B), V-2 (VIT 118A), and the proband, VI-1 (VIT118) are used for exome sequencing and *SMOC2* rs13208776.

Whole exome sequencing

DNA was quantified using the Qubit Broad Range assay on the Qubit 2.0 fluorometer(Thermofisher Scientific). Samples were then diluted to 100ng/µl and quantified using the Qubit High Sensitivity assay (Thermofisher Scientific). The libraries were then generated usingthe Ion Exome AmpliSeq Exome RDY kit (Thermofisher Scientific) according to the manufacturer's instructions. Libraries were quantified using the Tape Station (Thermofisher Scientific) or quantitative polymerase chain reaction (qPCR) (TaqMan, Quantistudio 3™, Thermofisher Scientific) and diluted to 100 ng/µl. The libraries were templated using the Ion Chef ™ system on 540 chips (Thermofisher Scientific). Two libraries were loaded per chip. Exomes generated from templating were then sequenced using the Ion Torrent S5 machine (Thermofisher Scientific).

Data analysis

After sequencing, the variant call format (VCFs) and the Binary Alignment/Map (BAM) files were downloaded from the Ion TorrentTM software (Thermorfisher Scientific). VCFs were uploaded and annotated using the Ion Torrent Reporter software (Thermorfisher Scientific). The Ion Torrent Reporter software filtered the variants according to the gene symbols of our interests which are *PTPN22*, *CTLA4*, *IKZF4*, *NLRP1*, and *TNFAIP3* (Table 1). The filtered VCFs were then downloaded from Ion Torrent Reporter (Thermofisher Scientific) and were uploaded and annotated on Ensembl Variant Effect Predictor (VEP) (GRCh37.p13). The annotated files from the VEP were downloaded in the TXT format and opened using MicrosoftExcel. The variants were filtered down on Microsoft excel according to the consequences (frameshift, nonsense, missense, and splice site mutations). The Integrative Genomics Viewer(IGV) software were used to visualize reads using BAM files to confirm the variants and assess the sequencing coverage and depth using the IGV 1.11.1 software.

Variants that met the filtering criteria were considered as those that could potentially lead to vitiligo and other AIDs. The filtering criteria were as follows; 1. variants in one of the five non-MHC genes (PTPN22, CTLA4, IKZF4, NLRP1, and TNFAIP3). 2. met the consequences criteria (frameshift, nonsense, missense, and splice site mutations). 3. had allele frequencies of or less than 0.01 or was novel. Variants were analyzed further to detect potentially functional variants and compared amongst the family members and across two families.

Table 1: non-MHC genes associated with Vitiligo and other autoimmune diseases analysed in this study

Non-MHC genes	Function	Reason	References
Protein tyrosine phosphatase nonreceptor type 22 *(PTPN22)*	Gives instructions for making a protein that belongs to the PTP (protein tyrosine phosphatases) family.	It has been associated with vitiligo, RA, T1D, SLE	Huraib et al., 2020 Garcia et al., 2014
IKAROS family zinc finger 4 *(IKZF4*)	It encodes the protein IKAROS that negatively regulates the immune system.	Has been associated with vitiligo and Alopecia `	Tang et al., 2012
Tumor necrosis factor-α–induced protein 3 *(TNFAIP3)*	Regulates crucial stages in immune cell homeostasis, such as NF-κB activation and apoptosis.	Has been associated with vitiligo, RA, SLE, and T1D	Martins et al., 2020 Fung et al., 2009
cytotoxic T-lymphocyte-associated protein 4 *(CTLA4)*	It functions as an immune checkpoint and downregulates the immune responses.	Has been associated with vitiligo and RA, MS, SLE, T1D	Birlea et al., 2009
NACHT leucine-rich-repeat protein 1 *(NLRP1)*	It encodes a member of the Ced-4 family of apoptosis proteins that help in protein-to-protein interaction formation.	Has been associated with vitiligo, psoriasis, SLE, RA	Alkhateeb et al., 2010 Jin et al., 2007

RA- rheumatoid arthritis; SLE- systemic lupus erythematosus; MS- multiple sclerosis; T1D- type 1 diabetes

S*MOC2* rs13208776 genotyping

Samples from both families were quantified and normalized to 20 ng/µl. The samples were then genotyped using TaqMan Assay (Thermofisher scientific) for SPARC Related Modular Calcium Binding 2 *(SMOC2*) SNP and were analyzed for genotypes using the Quantstudio 3 Software (Thermofisher Scientific).

Results

The DNA integrity, quality, and quantity were within the expected ranges. Libraries were generated and whole exome sequencing on nine DNA samples was successfully performed. The libraries were sequenced separately, however, they all produced good data which can be reliable as shown in Appendix A.

Thirty-eight variants from both Family 1 and 2 were found in the five selected genes (*TNFAIP3*, *NLRP1*, *PTPN22*, *CTLA4*, and *IKZF4*). Of the 38 variants identified, six were missense variants, two were frameshift variants, 16 were synonymous, four were intronic variants, two were 3- prime-UTR variants, two were regulatory variants, two were upstream gene variants, and four were splice NMD-transcript variant, further shown in Figure 3.

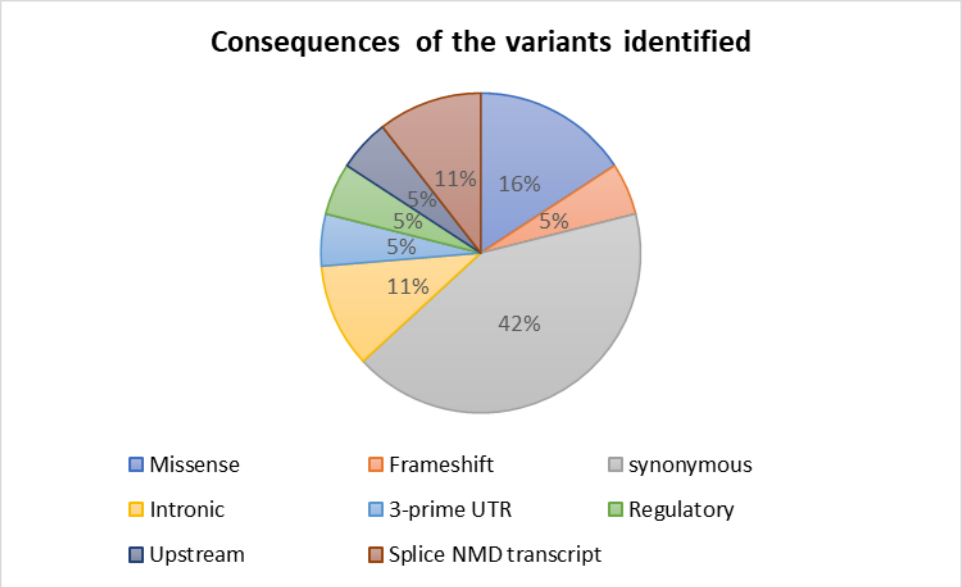
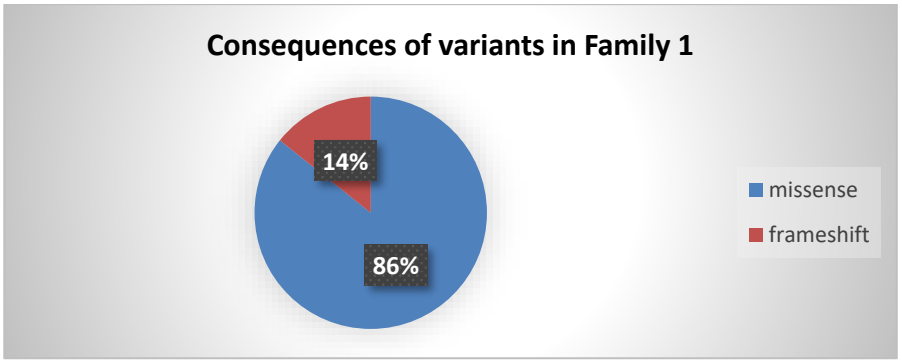


Figure 3: Consequences of the variants identified on five selected genes. Thirty-eight variants from all sequenced individuals in both Family 1 and 2 were identified before filtering down according to the selected consequences (missense, nonsense, frameshift, and splice site mutations).

The filtering criteria for the variants were: 1. variants in one of the five non-MHC genes (*PTPN22*, *CTLA4*, *IKZF4*, *NLRP1*, and *TNFAIP3)*. 2. met the consequences criteria (frameshift, nonsense, missense, and splice site mutations). 3. had allele frequencies of less than 0.01 or was novel. Eight variants that met our selection criteria of potential functional impact were seen in Family 1 and Family 2. Six were missense and two were frameshift variants (Figure 4). Of the selected five non-MHC genes, only 4 genes had variants that met the filtering criteria: *NLRP1*, *PTPN22*, *CTLA4*, and *IKZF4* (Table 2).

In Family 1, seven variants were identified in both affected and unaffected individuals. Six missense variants (of the six variants, three were common missense variants identified in affected individuals) and one frameshift variant was identified in unaffected individual of Family 1 (Figure 4a). Four variants were identified in individuals of Family 2. Three were missense variants and one was a frameshift variant seen on only the proband (Figure 4b). Three missense variants identified in affected individuals of Family 1 are the same missense variants also seen in Family 2 (Table 3).



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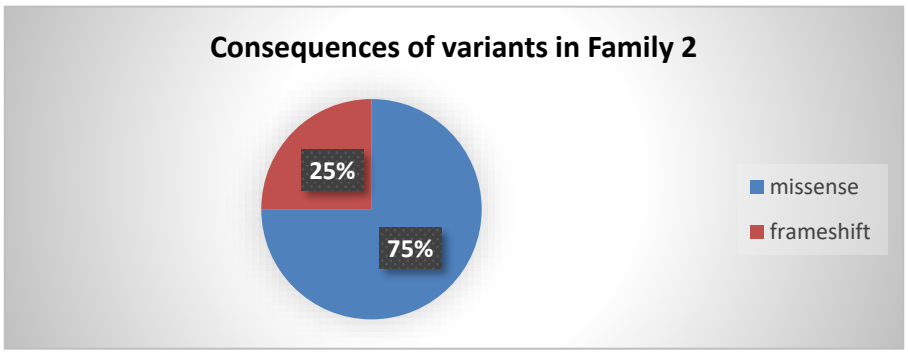


(b)

Figure 4: Consequences of variants identified in Family 1 and Family 2. **Five out of eight sequenced participants were from Family 1 and three from Family 2**. (a) Six variants seen in Family 1 were missense variants and one was a frameshift variant identified in an unaffected individual. Of the six missense variants, three were shared in all affected individuals and the other three missense variants were in unaffected individuals. (b) Four variants were identified in Family 2. Three were missense variants and one was a frameshift variant identified in only the proband.

Table 2: Variants, genotypes, and the consequences for individuals with and without autoimmune disorders of Family 1 and Family 2.

Variant	**Consequence**	**Genes**	**Co-ordinates**	**Family 1**						**Family 2**		
				III-1 (VIT113A)	III-12 (VIT113B)	**III-6 (VIT113C)**	III-2 (VIT113D)	II-2 (VIT113E)	**III-4 (VIT113G)**	**VI-1 (VIT118)**	**V-1 (VIT118A)**	**V-2 (VIT118B)**
rs11651270	Missense	*NLRP1*	17:5425077	**Y (C/T)**	N	**Y(C/T)**	N	N	**Y(C/T)**	**Y(C/T)**	**Y(C/T)**	**Y(C/T)**
rs2301582	Missense	*NLRP1*	17:5436263	**Y(T/C)**	N	**Y(T/C)**	N	N	**Y(T/C)**	**Y(T/C)**	**N**	**Y(T/C)**
rs12150220	Missense	*NLRP1*	17:5485367	**Y(T/A)**	N	**Y(T/A)**	N	N	**N**	**Y(T/A)**	**N**	**Y(T/A)**
rs114529583	Missense	*NLRP1*	17: 5467544	**N**	N	**N**	Y(G/A)	N	**N**	**N**	**N**	**N**
rs231775	Missense	*CTLA4*	2:2047391235	**N**	N	**N**	N	Y(A/T)	**N**	**N**	**N**	**N**
rs2476601	Missense	*PTPN22*	1:114377568	**N**	N	**N**	Y(C/T)	Y(C/T)	**N**	**N**	**N**	**N**
rs754906377	Frameshift	*PTPN22*	1:114391235	**N**	N	**N**	N	N	**N**	**Y(G/Deletion)**	**N**	**N**
ENST00000547791.1:c.317del	Frameshift	*IKZF4*	12:56420725	**N**	Y(C/Deletion)	**N**	N	N	**N**	**N**	**N**	**N**

Bolded are the individuals with no autoimmune disorders. Y- indicates that individuals have the variant, and N- indicates that individuals donot have the variant.

Table 3: Variants observed in both families

Variant	**Consequence**	**Gene**	**Family 1**	**Family 2**
rs11651270	Missense	*NLRP1*	3/6	3/3
rs2301582	Missense	*NLRP1*	3/6	2/3
rs12150220	Missense	*NLRP1*	2/6	2/3

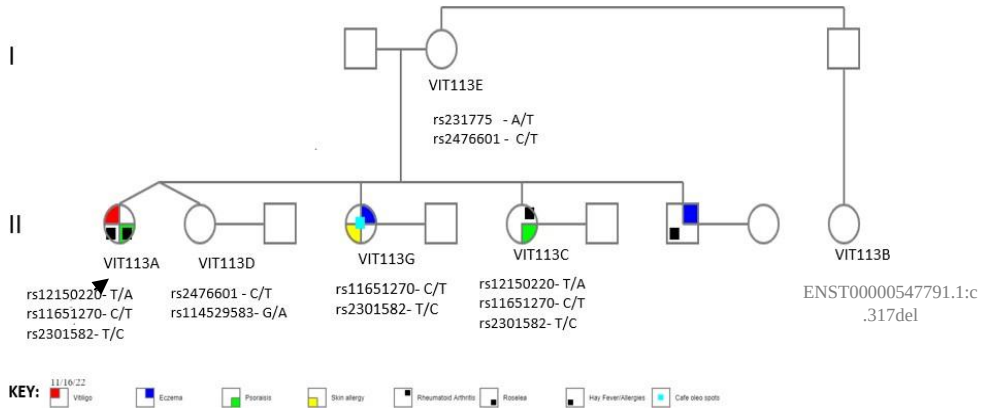
Variants identified in all affected individuals

Family 1

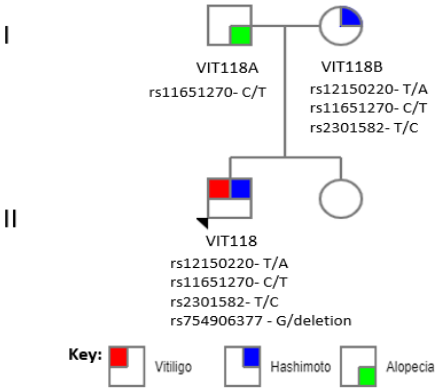
Individuals with vitiligo and /or autoimmune disorders (III-1 (VIT113A), III-4 (VIT113G), and III-6 (VIT113C) shared *NLRP1* variants (rs12150220, rs11651270, and rs2301582) and those without any autoimmune disorders did not have any variants that were seen in individuals with vitiligo and/or autoimmune disorders. Individual III-4 (VIT113G) has two variants, rs11651270 and rs2301582 and individual III-6 (VIT113C) has three variants, rs12150220, rs11651270, and rs2301582. These variants (rs12150220, rs11651270, and rs2301582) were shared amongst individuals with various autoimmune disorders including the proband. Genotypes for the variants are shown in the pedigree in Figure 5.

Family 2

The proband VI-1 (VIT118) has four variants, rs754906377, rs11651270, rs2301582 and rs12150220. The rs754906377 variant is in the *PTPN22* gene and is frameshift variant. The proband's mother V-2 (VIT118B) has rs11651270, rs2301582 and rs12150220 and the proband's father V-1 (VIT118A) has one variant, rs11651270 (Figure 5).



a.



b.

Figure 5: Family 1 and Family 2 pedigrees with genotypes in the four genes (*NLRP1*, *PTPN22*, *CTLA4*, and *IKZF4*) that met the selection criteria. (a) Pedigree of Family 1 with all sequenced individual's genotypes in the four genes. (b) Pedigree of Family 2 with the proband and his parents with genotypes in genes that met the selection criteria. *NLRP1* variants, rs12150220, rs11651270, rs114529583, and rs2301582, *PTPN22* variants rs2476601 and rs754906377, *CTLA4* variants rs231775 and *IKZF4* variant ENST00000547791.1:c.317del genotypes are shown in both figures.. Genotypes are shown under each individual.

Variants shared in both families

All identified variants that met the filtering criteria for a consequence are in Table 2. Three variants (rs12150220, rs11651270 and rs2301582) in the *NLRP1* gene were observed within both Family 1 and Family 2 in individuals with vitiligo and/or autoimmune disorders (figure 5).

Variants identified in Individuals without autoimmune disorders

The mother of Family 1's proband II-2 (VIT113E), the twin sister (non-identical, III-2 (VIT113D), and the cousin III-12 (VIT113B) are all unaffected individuals. They do not harbour any of the variants seen in any of the affected individuals (Figure 5a). The mother II-2 (VIT113E) and the non-identical twin sister have the rs2476601 variant located in the *PTPN22* gene, both in the heterozygous state. In addition, the mother also has the rs231775 variant located in the *CTLA4* gene in the heterozygous state. The probands cousin III-12 (VIT113B), who has a father with vitiligo but is herself unaffected, has a (novel) ENST00000547791.1:c.317del frameshift variant in the *IKZF4* gene.

***SMOC2* SNP genotyping results**

All genotyped individuals of Family 1 (nine individuals) were homozygous (A/A) for the SNP. In Family 2, the proband VI-1 (VIT118) and his mother V-2 (VIT118B) were heterozygous (A/G) and the father V-1 (VIT118A) was homozygous for allele 2 (A) as shown in Figure 6.

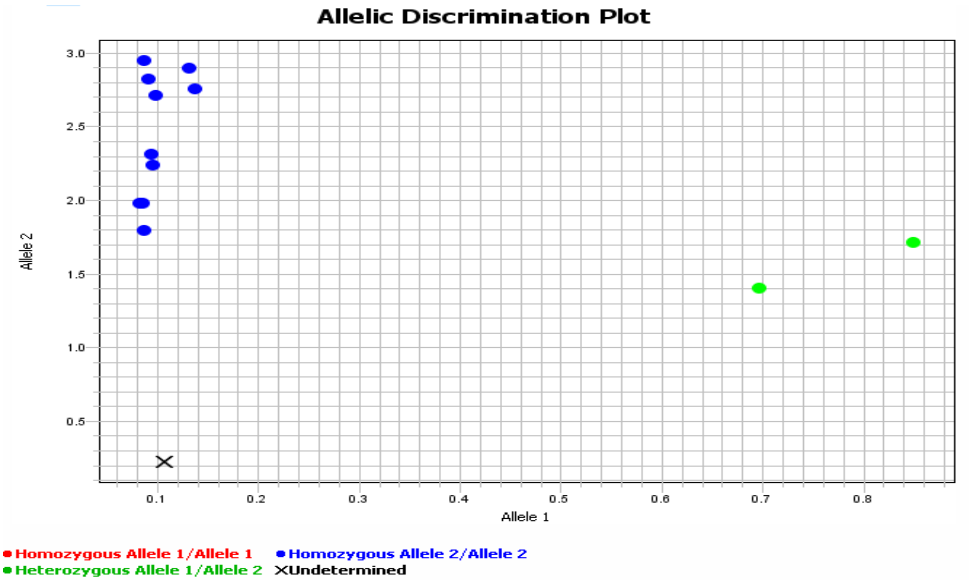


Figure 6: Allele discrimination plot for rs13208776 for Family 1 and Family 2. All individuals of Family 1 and the father (I-1(VIT118A) from family 2 were homozygous (A/A, blue circle) for the rs13208776 SNP. Individuals VI-1 (VIT118) and V-2 (VIT118B) were heterozygous (A/G, green circle (Allele 1/Allele 2).

Nine participants from two unrelated families of Indian descent were sequenced in this study. A total of eight variants that met our selection criteria were identified in both affected and unaffected individuals of both families. Four variants were found on the *NLRP1* gene, three were found in affected individuals while one variant was found an unaffected individual, and they were all missense mutations. Two

variants in affected and unaffected individuals were found in the *PTPN22* gene. A missense variant on the *CTLA4* gene and one frameshift variant on the *IKZF4* gene were also identified. A total of 12 individuals from two Indian families were genotyped for *SMOC2* SNP. All individuals of Family 1 were homozygous.

Discussion

This study aimed to identify genetic variants in selected non-MHC genes (*NLRP1, IKZF4, CTLA4, PTPN22,* and *TNFAIP3*) in individuals of Indian descent with vitiligo and their close relatives who have other autoimmune disorders or were unaffected using whole-exome sequencing. Most genetic studies have been conducted in European and East Asian (Chinese and Japanese) populations, with very few studies in Indian populations and none in African populations (Campbell et al., 2008, Popejoy et al., 2016) hence little is known about the genetics of vitiligo and its' genetic interactions with other autoimmune disorders in Indian and African populations. However, previous studies show that individuals with vitiligo often have other autoimmune disorders or first-degree relatives with at least one other autoimmune disorder (Alkhateeb et al., 2003, LaBerge et al., 2008). The pedigrees for Families 1 and 2 are suggestive of a shared mutation or mutations that are probably family-specific and show an autosomal dominant mode of inheritance with incomplete penetrance. The mutation/s is likely to be pleiotropic in that the same mutations cause different disorders in different people.

NLRP1 gene variants in affected individuals of Family 1 and Family 2

NLRP1 missense variants, rs12150220, rs2301582, and rs11651270, were found in affected individuals of both Family 1 and Family 2. *NLRP1* rs12150220 is a deleterious missense mutation variant that has been previously associated with vitiligo, systemic lupus erythematosus (SLE), and thyroid disease in a Norwegian population (Magitta et al., 2009). *NLRP1* rs12150220 was identified as a decreased risk polymorphism for autoimmune disorders associated with vitiligo such as T1D and SLE in the Caucasian population, in the United States by reducing the activity of the abnormal NLRP1 protein which could be because of its genotype A/T (Li et al., 2017). This genotype was not found to be associated with increased risks of developing vitiligo; however, the major allele A was found to be positively associated with a high risk of vitiligo- associated autoimmune disorders such as T1D, and SLE while the rare minor allele T is said to be protective against vitiligo (Li et al., 2017). The rs12150220 variant was found to have an allele frequency of 0.03 in both the Southern Asian population (Indian population) and the African population.

Missense *NLRP1* rs2301582 variant of mutation has a moderate impact and an allele frequency of 0.07 in the African population and 0.02 in the Asian (Indian) population. The rs2301582 variant was found to be associated with vitiligo phenotype and T1D, RA, psoriasis, pernicious anaemia, and thyroid disease (Hashimoto disease) in individuals of European descent from the United Kingdom and the United States (Jin et al., 2007).

Missense variant rs11651270 has been associated with autoinflammatory arthritis, dyskeratosis congenital (a rare telomere disease that results in bone marrow failure), and T1D in the Chinese Han population (Sun et al., 2019). Moreover, rs11651270 was associated with asthma in the Brazilian population (Leal et al., 2017). The rs11651270 variant was found with an allele frequency of 0.48 in the African and 0.27 in the Indian population.

The *NLRP1* gene is located on chromosome 17q13, consisting of 17 exons. It is considered a protein-encoding member of the Ced-4 family of apoptosis proteins which serves as a structural framework for protein-protein interaction formation (Alkhateeb et al., 2010). It is expressed at the highest level in the brain however, also in the skin cells, keratinocytes which plays a role in repairing skin and controlling cutaneous immune response (Kummer et al., 2007). NALP1 protein is also expressed at a high level in immune cells, particularly T cells and Langerhans' cells, which is consistent with *NALP1*'s involvement in skin autoimmunity (Jin et al., 2007).

It consists of three domains (NACHT domain, six leucine-rich repeats (LRR) domains, and function-to-find domain (FIIND), and two terminals (amino terminal PYD and carboxyterminal CARD) (Kummeret al., 2007). *NLRP1* gene also functions as an inflammasome, controlling the activation of protease caspase-1 which stimulates macrophages and monocytes (Kummer et al., 2007). Stimulation of macrophages and monocytes is associated with inflammatory responses which can result in various autoimmune disorders (Kummer et al., 2007). *NLRP1* variants found in affected individuals in this current study may be inherited and segregate together as a haplotype and be co-associated with different disorders. Moreover, they could be pleiotropic, causing different disorders in different individuals.

***NLRP1* rs114529583 found on the unaffected individual of Family 1**

NLRP1 rs114529583, a missense variant found in the unaffected twin sister (III-2 (VIT113D) of the proband (III-1 (VIT113A) in Family 1 has not been associated with any phenotypes, however, it has an

allele frequency of 0.01 in the African population and 0.0 in the Indian population. This shows that the variant may be rare but not cause any disorder.

PTPN22 gene Variants found on the affected proband of Family 2 and unaffected individuals of Family 1

The missense variant rs2476601 was found in non-affected individuals of Family 1 (the proband's non-identical twin sister, III-2 (VIT113D), and their mother II-2 (VIT113E). The variant changes the nucleic acid cytosine to thymidine (C1858T) changing the amino acid Arginine to Tryptophan (Huraib et al., 2020).

PTPN22 rs2476601 has been associated with RA, SLE, and T1D in the European population from the United States and the United Kingdom (Saevarsdottir et al., 2020) and with vitiligo in individuals of European descent from the United Kingdom (LaBerge et al., 2008). This variant, rs2476601 was also found to be associated with vitiligo in the European population, United Kingdom, however, it was only found as a risk factor for vitiligo in Romanian, North American, South Indian Tamin, and Mexican populations but it was not found to be associated with vitiligo or as a risk factor for vitiligo in the Asian population (Hurabet al., 2020). Furthermore, *PTPN22* rs2476601 was not significantly associated with vitiligo in Egyptian females (Elmongy et al., 2013) and Jordanian (Alkhateeb et al., 2010), Turkish, and Indian Gujarat populations (Laddha et al., 2008). These contradictions of the *PTPN22* gene variant in the literature indicate that ethnic groups play a role in the susceptibility (Huraibet al., 2020), yet it was identified in unaffected individuals.

This rs2476601 variant is common in the Indian population with the allele frequency of 0.48. This adds the knowledge that the rs2476601 variant is basically common in Indian populations. rs754906377, a missense variant with an allele frequency of 0.27 in the Indian population was found in the affected proband of Family 2 VI-1 (VIT118), however, it has not been associated with any phenotype.

The *PTPN22* gene contributes to the genetic risk associated with autoimmune disorders including vitiligo (LaBerge et al., 2008, Tizaoui et al., 2021) by negatively regulating the T cell receptor which regulates the innate immune system (Bottini et al., 2014) through tyrosine phosphatase protein it encodes. This protein increases the activity of the LYP protein which then inhibits the signaling of the T Cell resulting in autoimmunity (Huraib et al., 2020). This protein is expressed by the cells of the

immune system (lymphocytes -T cells and B cells) thus influencing the immune malfunction and ultimately autoimmunity (Tizaoui et al., 2021). In Colombian families, the *PTPN22* rs2476601 was associated with T1D (Rodríguez et al., 2015). The *PTPN22* gene is a convincing gene for the contribution towards autoimmune disorders yet the variants identified in this gene were identified in unaffected individuals.

CTLA4 variant found in the unaffected individual in Family 1

CTLA4 had a missense rs231775 variant found in unaffected mother II-2 (VIT113E) of the proband of Family 1. VIT113E was heterozygous for the with the very rare T allele that has only been observed in East Asian at a very frequency (5.442e-05) (Auton et al., 2015). This allele has not been associated with any phenotypes as it is ultra-rare. However, the more common variant allele (G) not found to be associated with any autoimmune disorder in individuals of European descent of the United States (LaBerge et al., 2008) but found to be associated with Graves' disease in Han Chinese (Tu et al., 2017). The rs231775 T variant was found in an unaffected individual and not in any affected individuals in this current study and there is no evidence of this very rare allele being associated with any phenotypes thus it is unlikely to be involved in disease susceptibility in individuals of Indian descent.

A novel variant found in the _IKZF4_ gene in an unaffected individual in Family 1

A novel frameshift mutation (ENST00000547791.1:c.317del) was identified an unaffected individual (III-12 (VIT113B)) in Family 1 on the *IKZF4* gene. This gene produces a transcription factor, EOS protein acts as a T cell-negative regulator of the immune system (Miao et al., 2022). Variants in this gene could lead to a lack of protein production which could result in autoimmunity (Miao et al., 2022). *IKZF4* was strongly associated with early childhood T1D (Lempainen et al., 2013).

No variant in the _TNFAIP3_ gene that met the filtering criteria

Variants in the *TNFAIP3* gene in our participants did not meet the selection criteria consequences. This gene encodes an enzyme that negatively regulates inflammation (Lodolce et al., 2010). Unregulated inflammation leads to damaged cells which can resultin autoimmunity. Previously identified variants in the *TNFAIP3* gene, rs6920220 and rs1800972 are risk polymorphisms for vitiligo in Egyptian patients (Saleh et al., 2022).

Major allele SMOC2 SNP found in most individuals

The *SMOC2* gene encodes an extracellular calcium-binding glycoprotein with an unknown function (Shen et al., 2016), however, the *SMOC2* SNP rs13208776 has been linked with vitiligo in European people of north Romania and it was also found to be associated with RA and T1D (Birlea et al., 2010). From our study, we observed the minor allele of *SMOC2* SNP in two individuals of the same family. Thus, it is highly unlikely to contribute to the vitiligo phenotype or other immune disorders because all the individuals that have vitiligo have not been tracked with this allele.

Autoimmune disorders are caused by both genetic and environmental components (LaBerge et al., 2008). All variants are found on genes that respond to the immune system, and the genes are also shown to be the common genetic loci for immune disorders (Martins et al., 2020). Moreover, we sequenced non-identical twin sisters whom we assume grew up in the same environment but one (the proband) has vitiligo and other autoimmune disorders while the twin sister does not have any autoimmune disorders. This suggests that manifestation of the proband's disorders was mainly influenced by her genetics, which have been associated with vitiligo and other autoimmune disorders. Our results showed common variants within family members with vitiligo and/or various autoimmune disorders. The variants could be pleiotropic hence, the same variants can result in various autoimmune disorders in different individuals, or the identified variants have nothing to do with the development of autoimmune disorders.

Limitations

The study only had nine participants which is a relatively small sample size. Thus, it is limited by the study sample size. Noncoding regions were not sequenced because our study was only looking at coding regions. Additionally, only five non-MHC genes were analysed and if variants in other genes were present and contribute to disease, they were missed. Due to time constraints, variants were not validated using Sanger sequencing. The SMOC2 SNP was genotyped using the TaqMan genotyping assay. Although this assay is very accurate, there is a small chance genotypes may be called incorrectly using this protocol.

Haplotype analysis allows for grouping of a set of variants that are often inherited together. Three SNPs in the NLRP1 gene found in all individuals with an autoimmune disorder in both families. These SNPs may form haplotypes, but this was not assessed in this study. Thus, assessing the co-segregation of haplotypes with autoimmune disorders could have been informative as to whether haplotypes in the NRPL1 gene segregate with autoimmune disorders.

Conclusion

We sequenced nine participants from two unrelated families of Indian descent. A total of eight variants that met our selection criteria were identified in both affected and unaffected individuals of both families. Four variants were found on the NLRP1 gene, three were found in affected individuals while one variant was found on unaffected, and they were all missense mutations. Two variants in affected and unaffected individuals were found in the PTPN22 gene. A missense variant on the CTLA4 gene and one frameshift variant on the IKZF4 gene were also identified.

Variants that met our selection criteria were found in 4 out of 5 genes we had selected. The potentially pathogenic variants in Family 1 were not passed down from the mother as we had hypothesized, but in Family 2, they seem to have been passed from the parents to the child, suggesting that the mutations are pleiotropic. This study showed that individuals with various autoimmune disorders have common variants in the *NLRP1* gene suggesting that the *NLRP1* variants may have segregated with vitiligo and other autoimmune disorders within family members. In *SMOC2* SNP genotyping, we managed to genotype and analyse individuals that are heterozygous and homozygous (A/A) for the SNP rs13208776. The *SMOC2* effect allele was not passed down with vitiligo or autoimmune disease in family 1 suggesting that it is unlikely to contribute to susceptibility for vitiligo in this Indian family, however, further research in a larger Indian population is required as it will be more effective and enhance the statistics data.

Only five genes were investigated in two families. However, exome sequencing was conducted in this study therefore variant data from other genes is available for analysis. For future studies, other genes could be analysed to identify any coding variants that segregate with the disorders in each or both families. Additionally, more families could be added to the study to increase the size.

Conflict of Interest

None to declare

Author Contributions

Kentie Rabinda: Performed the bulk of the lab work and analysis and interpretation of the results. Drafted the manuscript

Thnadiswa Ngcungcu: conception of the work, performed some of the lab work. Helped with the analysis and interpretation of findings. Contributed to the drafting of the manuscript.

Acknowledgements

Dr Lushen Pillay for assessing and diagnosing the participants, Dr Lindie Lamola for assistance in the laboratory.

Statement of contribution to the field

Individuals with vitiligo usually have one other autoimmune disorder or first-degree relatives with autoimmune disorders. There is genetic overlap for vitiligo and other autoimmune disorders in major histocompatibility genes and non-major histocompatibility complex genes. This study showed that specific variants in the *NRPL1* gene are present in families of Indian descent that are affected by vitiligo and other autoimmune disorders.

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Appendices

Appendix A

A plugin summary showing the quality of the sequencing run of all samples (sequenced) and the number of variants produced.

Sequencing plugin summary Table

Run no.	VIT ID	Variants	>_Q20	ISP loading (%)	Usable sequence (%)	Polyclonal (%)
1	III-1(VIT113A)) III- 6(VIT113C)	21,479 26,324	3,045,194,211 6,718,923,558	89	43	45
2	II-2(VIT113E)	36,681	7,675,610,762	92	68	27
3	III-4(VIT113G) V-1(VIT118A)	15,511 25,425	2,565,802,181 4,001,626,653	82	32	49
4	III-12(VIT113B)	33,125	879,015,184	81	48	33
5	VI-1(VIT118)	33,449	8,568,754,449	78	45	29
6	III-2(VIT113D) V-2(VIT118B)	33,577 30,970	7,347,185,930 6,007,704,709	94	56	39

Appendix B

Research Protocol

<table>
<tr><td>CANDIDATE'S

SURNAME:
Rabinda</td><td>FIRST
NAMES:
Kentie
Rofhiwa</td><td>STUDENT
NUMBER:
1132300</td></tr>
<tr><td colspan="3">CURRENT

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<tr><td colspan="4">DEGREE FOR WHICH PROTOCOL IS BEING SUBMITTED: MSc (Med) Genomic Medicine</td></tr>
<tr><td colspan="4">PART-TIME OR FULL-TIME: Full time:</td></tr>
<tr><td colspan="2">FIRST REGISTERED FOR THIS DEGREE:</td><td>TERM: January</td><td>YEAR: 2022</td></tr>
<tr><td colspan="4">DEPARTMENT: Division of Human Genetics</td></tr>
<tr><td colspan="4">TITLE OF PROPOSED RESEARCH: Exome Sequencing of individuals with vitiligo and their relatives with and without autoimmune disorders</td></tr>
<tr><td colspan="3">CANDIDATE'S SIGNATURE:  </td><td>DATE: 26 April 2022</td></tr>
<tr><td colspan="3">SUPERVISOR 1 (NAME & SURNAME): Thandiswa Ngcungcu</td><td>% Supervision 100</td></tr>
<tr><td colspan="4">SUPERVISOR'S QUALIFICATIONS Ph.D., MSc (Med), BSc (Hons), BSc</td></tr>
<tr><td colspan="4">SUPERVISOR'S DEPARTMENT Division of Human Genetics</td></tr>
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WITS ETHICS NOT REQUIRED: Yes No WITS ETHICS PENDING: No WITS ETHICS APPROVED: Yes (circle appropriate symbol)* ***Please note the final human ethics clearance certificate or animal ethics certificate must be available prior to starting research**	IF Y SUPPLY ETHICS CLEARANCE CERTIFICATE AS ATTACHMENT AND INCLUDE ETHICS NUMBER HERE: M220389

As supervisor/s, I/we confirm that I have read the protocol which has been submitted for assessment.

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SYNOPSIS OF RESEARCH CONTINUED

Autoimmune disorders result from the immune system attacking and damaging its own healthy cells because ofan acquired immune system malfunction. Hashimoto's disease, psoriasis, rheumatoid arthritis including vitiligo are examples of autoimmune disorders. Vitiligo is a common depigmentation skin disorder with an estimated prevalence of 0.5–2% of the population worldwide. The disease is characterized by the selective loss of melanocytes which results in non-scaly, chalky-white macules and is classified into two types, nonsegmental vitiligo and segmental vitiligo based on the distribution of vitiligo lesions across the body. All ethnic groups and both adults and children can be affected by vitiligo. Vitiligo results from complex interactions between several genetic and environmental factors. Individuals with vitiligo or some first-degree relatives often have at least oneother autoimmune disorder.

Genome-wide association studies (GWAS) identified 50 genetic loci that confer risk for vitiligo, and many of these loci are associated with other autoimmune diseases. Polymorphisms in *HLA-A* confer the most significantgenetic risk of vitiligo and other risk factors for vitiligo also relate to antigen presentation (*HLA-DRB1/DQA1*, *CPVL*). *HLA* genes directly present antigen and *CPVL* is a peptidase involved in antigen processing. Genetic polymorphisms likely predispose patients to autoimmune dysregulation, and melanocyte abnormalities, they initiate pathologic killing of melanocytes. Environmental triggers then act upon genetically predisposed individuals to help initiate and amplify vitiligo. The aim of this study is to identify genetic variants in selected genes in individuals with vitiligo and their unaffected close relatives who have other autoimmune disorders. Fivenon-MHC genes are selected for analysis. DNA will be extracted. Exome sequencing will be performed, and bioinformatics will be performed to annotate identified variants. Potential variants will be compared between individuals with vitiligo and their close relatives with and without autoimmune disorders in a family-specific manner.

Exome sequencing of individuals with vitiligo and their relatives with and without autoimmune disorders

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Contents

1. Introduction

Autoimmune disorders result from the immune system attacking and damaging its healthy cells because of an acquired immune system malfunction (Frisoli et al., 2020). Autoimmunedisorders are caused by a complex interaction of genetic and environmental factors (Narita et al., 2011). Autoimmune disorders are the 3rd most common disease category after heart diseases and cancer, they have prevalence of 8% in the general population with a high incidence in women (Ezzedine et al., 2015). The onset of autoimmune disorders is usually during childbearing years for women, which is between 14 – 44 years (Ramos et al., 2015).

Autoimmune disorders are distinguished by the presence of antibodies that attack specific self-cells by binding to their receptors, leading to cell lysis and, eventually, disease pathogenesis (Iannella et al., 2016). Antibody-mediated cell lysis also involves macrophages(Baldini et al., 2017). Macrophages are long-lived cells that use phagocytosis to provide significant security against aggressive events (Iannella et al., 2016).

Hashimoto's disease, psoriasis, rheumatoid arthritis, and vitiligo are examples of autoimmune disorders (Baldini et al., 2017). Co-occurrence of at least two autoimmune disorders in people who have an autoimmune disorder and/or their first-degree relatives (Richard-Eaglin et .al, 2018) has been observed. For example, people with vitiligo, a skin depigmentation autoimmune disorder, usually have another autoimmune disorder or their close relatives have one or more autoimmune disorders (Baldini et al., 2017). This suggests that there are common genetic factors underlying the pathogenesis of autoimmune disorders.

1.1 Genetics of autoimmune disorders

Autoimmune disorders have a common predisposing genetic background that leads to autoimmunity (Gill et al., 2016). The genetic cause or susceptibly for many autoimmune disorders has been linked to genes in the major histocompatibility complex (MHC) but non- MHC genes have also been implicated in susceptibility for autoimmune disorders (Iannella etal., 2016).

2. Vitiligo

Vitiligo is a skin disorder, caused by the loss of melanocytes which leads in depigmentation of the affected areas of the skin (Ezzedine et al., 2011). Melanocytes are the skin cells that produce melanin protein, that gives the skin colour (Frisoli et al., 2020). Despite the absenceof physical discomfort such as pain, vitiligo can have a negative impact on patients causing mental health issues affecting their self-esteem and affected individuals experiencing depression (Rasagna et al., 2019).

Vitiligo is clinically classified into two main types, segmental vitiligo (SV) and non-segmental vitiligo (NSV), based on the distribution of vitiligo lesions across the body (Baldini et al., 2017)and an additional type called unclassified vitiligo. SV has unilateral depigmentation that doesnot cross the midline of the body whereas NSV has bilateral depigmentation, it spreads all over the body (Ganapathy et al., 2017). SV has an earlier age of onset as compared to NSV (Ezzedine et al., 2011). Each of these types have subtypes. NSV, also called generalized vitiligo (the most common type of vitiligo) includes vitiligo vulgaris, vitiligo universals, and acrofacial vitiligo (Ezzedine et al., 2011, Baldini et al., 2017). SV includes focal vitiligo and mucosal vitiligo (Ezzedine et al., 2011, Baldini et al., 2017). The dermal phenotypic expression difference between vitiligo subtypes is critical for prognosis and determining the treatment approaches (Spritz & Santorico, 2021). Segmental vitiligo onset, natural course, and response differ from non-segmental vitiligo (Iannella et al., 2016).

Vitiligo is the most common skin disorder, affecting approximately 70 million people worldwide (Iannella et al., 2016). Vitiligo has a prevalence of approximately 0.5-2% of the population in adults and children worldwide (Iannella et al., 2016). Vitiligo affects all ethnicsgroups. The average age of onset of vitiligo is 30 years of age (Narita et al., 2011).

2.1. Vitiligo and other autoimmune diseases

Patients' first-degree relatives have higher rates of vitiligo and autoimmune disorders than the general population (Gill et al., 2016). At least 30% of patients with vitiligo were found to have other autoimmune disorders (Iannella et al., 2016). Several studies of vitiligo patients from different populations have revealed a strong epidemiological association with several other autoimmune disorders particularly autoimmune thyroid disease (Hashimoto’s disease),rheumatoid arthritis, adult-onset type 1 diabetes mellitus, psoriasis, pernicious anaemia, and Addison’s diseases to name a few (Gill et al., 2016; Narita et al., 2011).

2.2 Causes of vitiligo

Vitiligo results from selective loss of melanocytes which leads to depigmentation of skin withchalky white macules (Frisoli et al., 2020). Melanocyte death in vitiligo is mainly caused by self-reactive immune function (Frisoli et al., 2020). An autoimmune reaction is usually triggered or enhanced by the abnormal T helper and incompetent regulatory T cells of innateimmunity and CD8+ cytotoxic T lymphocytes eventually kill melanocytes, which result in vitiligo (Frisoli et al., 2020). This reaction is triggered by environmental stimuli, but genetic factors are said to account for a larger proportion of the risk of developing vitiligo (Roberts etal., 2019).

Vitiligo is caused by the complex interplay between genetic and environmental factors.

Ultraviolet (UV) radiation, pollution, microorganisms together with metabolic and oxidative stress and cell detachment abnormalities are examples of environmental causes (Frisoli etal., 2020).

2.2.1. Genetics of vitiligo

Vitiligo has a high heritability of 50-85% across different populations (Roberts et al., 2019). Italso has a modest concordance where patients' first-degree relatives have higher rates of vitiligo and autoimmune disorders with 6% in siblings and 23% in identical twins of Europeandescent (Iannella et al., 2016). Common genetic variants account for 70% of the genetic riskwith the development of vitiligo while rare genetic variants account for 30% of the genetic risk with the development of vitiligo (Narita et al., 2011).

2.2.1.1 Genome-wide linkage and candidate gene association studies

Autoimmune susceptibility loci (AIS) association with vitiligo have been linked with vitiligo through genome-wide linkage analysis. *AIS1* and *AIS2* are also involved T cell receptor repertoire and immune cell associated apoptosis which indicates the critical effects of abnormal immune system has in vitiligo pathogenesis (Chang et al., 2017).

2.2.1.2 Genome-wide association studies

Genetic polymorphisms likely predispose patients to autoimmune dysregulation, and melanocyte abnormalities (Chen et al., 2021). Genome-wide association studies (GWAS) identified approximately 50 genetic loci that confer risk for vitiligo, and many of these loci areassociated with other autoimmune diseases (Roberts et al., 2019). Polymorphisms in Humanleukocyte antigen A (*HLA-A)* confer the most significant genetic risk of vitiligo and other risk factors for vitiligo also relate to antigen presentation (Shen et al., 2016). Some of the MHC and non-MHC genes associated with vitiligo and other autoimmune disorders identified by GWAS are shown in table 1 below.

Table 1: Selected MHC and Non-MHC genes associated with autoimmune disorders.

Disorders	MHC genes	Non-MHC genes	References
Vitiligo, Systemic Lupus Erythematosus, Rheumatoid Arthritis	DDR1	PTPN22	Silva et al., 2010 and Huraib et al.,2020
Vitiligo, rheumatoid arthritis, SLE	XBP1	NALP1	Birlea et al., 2011 and Alkhateeb et al., 2010
Vitiligo, Gravesdisease, Systemic lupus erythematosus, Type I diabetes, rheumatoid arthritis	HLA - A	CTLA4	Xi et al., 2018 and Birlea S et al., 2009
Vitiligo, Rheumatoid arthritis	TSLP	TNFAIP3	Birlea et al., 2011, Martins et al., 2020 and Markovic et al., 2020

DDR1- discoidin domain receptor tyrosine kinase 1, *PTPN22*- Protein tyrosine phosphatasenon-receptor type 22, *XBP1*- X-box binding protein 1, *NALP1*- NACHT leucine-rich-repeat protein 1, *HLA-A*- human leukocyte antigens A, *CTLA4*- cytotoxic T-lymphocyte-associated protein 4, *TSLP*- Thymic Stromal Lymphopoietin, *TNFAIP3*- TNF Alpha Induced Protein 3

2.2.1.3 Non-major histocompatibility complex genes to be analysed in this study

Five non-major histocompatibility complex (non-MHC) genes will be analysed for mutationsthat might be causative for vitiligo and other autoimmune disorders present in the two families that will be included in the current study.

The Protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) gene is located on chromosome 1p13 and provides instructions for making a protein that belongs to the PTP (protein tyrosine phosphatases) family (Huraib et al., 2020). PTP protein regulates the cell signals (Huraib et al., 2020). *PTPN22* SNP rs2476601 has been associated with the development of vitiligo with GWAS in individuals of European descent and has been reportedto have a strong association with graves' disease, psoriasis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), diabetes type 1 and Hashimoto's disease (Garcia et al.,2014). IKAROS family zinc finger 4 *(IKZF4)* gene located in 12q13 chromosome which encodes the Ikaros protein that regulates the development of the immune cells (Tang et al., 2012). I*KZF4* has been associated with vitiligo in Chinese Han population and GWAS found rs10876864 SNP to be strongly associated with type 1 diabetes and alopecia (Tang et al., 2012).

Tumor necrosis factor-α–induced protein 3 *(TNFAIP3)* located on chromosome 12q24, regulates crucial stages in immune cell homeostasis, such as necrosis factor kB (NF-kB) activation and apoptosis (Martins et al., 2020). *TNFAIP3* is associated with vitiligo, RA, SLE,T1D locus (Fung et al., 2009). The cytotoxic T-lymphocyte-associated protein 4 *(CTLA4)* located at 2q33 chromosome functions as an immune checkpoint and downregulates immune responses (Birlea, S et al., 2009). *CTLA4* rs231775 SNP has been associated with

thyroid disease, psoriasis, SLE, alopecia and T1D (Birlea, et al., 2009). A meta-analysisshowed that that rs231775 SNP is associated with vitiligo in individuals with other autoimmune disorders (Birlea, et al., 2009).

The NACHT leucine-rich repeat protein 1 *(NALP1)* gene is located on chromosome 17q13 and encodes a member of the Ced-4 family of apoptosis proteins which serves a structural framework for protein-protein interaction formation (Alkhateeb et al., 2010). *NALP1* gene is associated with RA, psoriasis, pernicious anaemia, SLE and Addison disease (Jin et al., 2007). *NALP1* was found to have association with the development of vitiligo in individuals ofEuropean descent (Jin et al., 2007). The rs1008588 SNP, located on the promoter region of the *NALP1* gene was also found to be associated with vitiligo in Jordanians (Jin et al., 2007).

2.2.1.4 *SMOC2* gene association with vitiligo

SPARC Related Modular Calcium Binding 2 *(SMOC2),* a gene located on 6q27, was identified as a risk locus for vitiligo in a GWAS in individuals from two adjacent Romanian isolate villages with vitiligo and other autoimmune disorders (Birlea, et al., 2010). The *SMOC2* protein is localised in the basement layer of the epidermis, and it stimulates the attachment of primary keratinocytes (Birlea, et al., 2010). The *SMOC2* rs13208776 variant, located in intron 4 of the *SMOC2* gene (Shen et al., 2016), was associated with vitiligo in Romanians (Birlea, et al., 2010). However, this association was not replicated in Jordanians(Shen et al., 2016). The rs13208776 SNP showed marginal association with Hashimoto's disease in Jordanian women (Alkhateeb, et al., 2013).

3. Aims and Objectives

The aim of study is to identify genetic variants in selected non-MHC genes of individuals withvitiligo and their unaffected close relatives who have other autoimmune disorders. This will beachieved through the following objectives:

1. Extract DNA from venous blood samples of eight individuals
2. Perform exome sequencing
   2.1. Perform bioinformatics analysis to annotate identified variants
   2.2. Identify potential variants in the exome, present in affected individuals (vitiligo and other autoimmune disorders)

3. Genotype the *SMOC2* rs13208776 SNP using the TaqMan assay to determine if this variantin present in our South African families of Indian descent affected by vitiligo and other autoimmune disorders.

4. Methods

4.1 Study participants

Eight individuals of Indian descent will be subjected to whole exome sequencing. The study iscomprised of eight participants from two families with vitiligo across multiple generations.

Five individuals from Family 1 (Figure 2.1, complete pedigree in appendix A), one with vitiligo individual (VIT113A, proband), her unaffected mother (VIT113E), cousin (VIT113B), her fatherhas vitiligo) along with her two sisters, one who has psoriasis and rheumatoid arthritis (VIT13C) and the other with eczema, cafe au lait spots and unclassified skin allergies (VIT113G). A nuclear family (Family 2) with a male proband with vitiligo and Hashimoto disease, his mother who has Hashimoto disease and his father who has alopecia will also beincluded. The eight individuals and an additional three individuals from family 1 will be genotyped for the *SMOC2* rs13208776 SNP.

4.2. DNA extraction

Venous blood was collected from each participant. DNA will be extracted using the salting out method (Miller et al., 1988). The Genfind V3 50 prep kit method (Seipp, et al., 2010) will be used to extract samples that would not have been successfully extracted with salting out.The DNA quality will be assessed using gel electrophoresis (low percentage gel, 0.8%). Thequality and quantity (concentration of DNA per µl) of the DNA will be analysed using the Nanodrop™2000 (ThermoFisher Scientific), to determine the A260/A280 and A260/A230 ratios to determine levels of protein and RNA contamination, respectively.

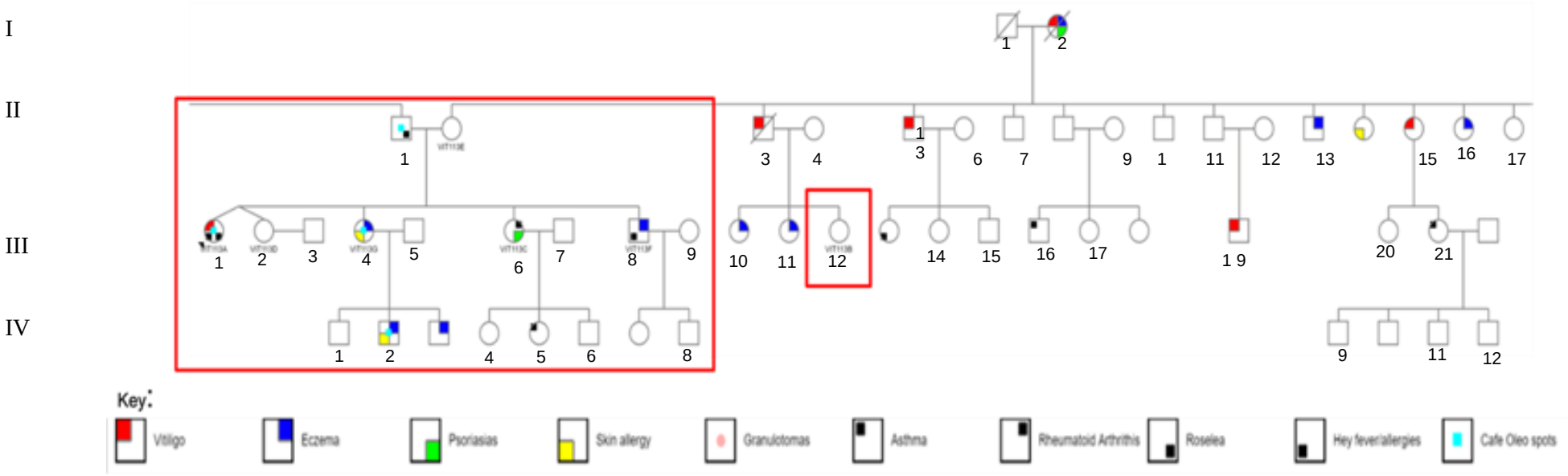


Figure 1: Pedigree of family 1. The probands' maternal side shows multigenerational tracking of vitiligo and various autoimmune diseases. The individuals with a VIT ID within the red blocks will be used in this study. Individuals II-2 (VIT 113E), III-1 (VIT 113A), IIII-4 (VIT 113G), III-6 (VIT 113C), III-12 (VIT 113B) will be used for exome sequencing and in addition to these individuals, III-2 (VIT113D), III-8 (VIT113F) will be genotyped for the *SMOC2* rs13208776 SNP.

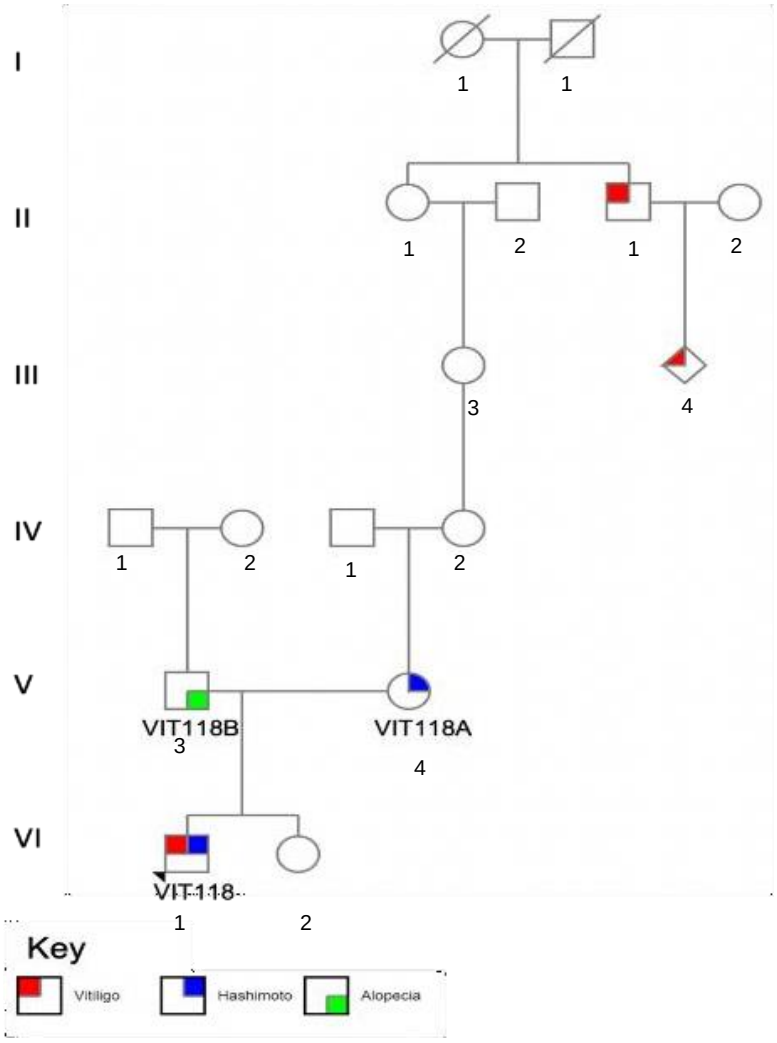


Figure 2: Pedigree of family 2. The probands' maternal side shows multigenerational tracking of vitiligo. The individuals with a VIT ID will be used in this study. Individuals V-1 (VIT18B), V-2 (VIT 118A), VI-1 (VIT118) will be used for exome sequencing and *SMOC2*rs13208776 SNP genotyping.

4.3 Whole exome sequencing

Whole exome sequencing (WES) is an application of the next generation sequence technology that sequences in the coding regions (exons) genome (Chang et al., 2017). When generatingthe libraries, the genomic DNA is fragmented into smaller fragments. Adaptors and barcodesare added to the fragmented DNA. Exons are then selected through target selection. The exonlibraries are then sequenced. Library preparation for WES will be done using Ion Exome AmpliSeq Exome RDY kit. Exomes will then be sequenced for the individuals listed in section4.1, using Ion Torrent S5 (in-house) and analyse the sequence using bioinformatics tool.

4.3.1. Non-MHC genes to be analysed tabulated

Table 2 below shows the selected non-MHC genes associated with vitiligo and other autoimmune disorders; the selected genes will be analysed for variants causing autoimmunedisorders. Due to time restriction, the entire exome will not be analysed in this project, and we have opted to anlayse non-MHC genes because the MHC may be poorly sequenced dueto the genetic complexity (high number of repeats) and difficulty in sequencing the region. A literature search has been conducted to select at least five appropriate genes that have beenassociated with vitiligo and other autoimmune disorders. Due to the difficulty of sequencing the MHC region, only non-MHC genes were selected for analysis.

Table 2: non-MHC genes associated with Vitiligo and other autoimmune diseases tobe analysed in this study

Location	Non-MHC genes	Function	Disorder associated with gene	References
1p13.2	Protein tyrosine phosphatase non receptor type 22 *(PTPN22)*	provides instructions for making a protein that belongs to the PTP (protein tyrosine phosphatases) family.	It has been associated with vitiligo, RA, T1D, SLE	Huraib et al., 2020 and Garcia et al., 2014
12q13.2	IKAROS family zinc finger 4 *IKZF4*	Encodes protein IKAROS	Has been associated with vitiligo and Alopecia `	Tang et al., 2012
12q24.13	Tumor necrosis factor-α–induced protein 3 *(TNFAIP3)*	regulates crucial stages in immune cell homeostasis, such as NF-κB activation and apoptosis	Has been associated with vitiligo, RA, SLE, and T1D	Martins et al., 2020 and Fung et al.,2009
2q33.2	cytotoxic T-lymphocyte-associated protein 4*(CTLA4)*	functions as an immune checkpoint and downregulates immune responses.	Has been associated with vitiligo and RA, MS, SLE, T1D	Birlea, S et al., 2009
17p13	NACHT leucine-rich-repeat protein 1 *(NALP1)*	This *gene* encodes a member of the Ced-4 familyof apoptosis proteins	Has been associated with vitiligo, psoriasis, SLE, RA	Alkhateeb et al., 2010 and Jin et al., 2007

SLE- Systemic Lupus Erythematosus; RA- Rheumatoid Arthritis; T1D- Type 1 Diabetes; MS-Multiple Sclerosis

4.3.2 Analysis strategy

Vitiligo and other autoimmune disorders are multifactorial disorders with many genes accounting for the large portion of the genetic cause for the disorders. However, the pedigreesfor Families 1 and 2 are suggestive of a common mutation or mutations that are probably family-specific, show an autosomal dominant mode of inheritance with incomplete penetrance.

The mutation/s is likely to be pleiotropic in that the same mutations causes different disordersin different people. This mode of inheritance is hypothesised in these two families and will beused to initially filter variants.

Variants in the selected genes will be extracted with the Ion Reporter tool that is available onthe sequencing platform. Variants will be identified, annotated, and analysed. Variant annotation will be done using Ensembl's Variant Effect Predictor (VEP) and the annotation file will be filtered for a variant consequence where frameshift, nonsense, missense, and splice site variants will be prioritised. Additionally, variants will be filtered by allele frequency in Indianpopulations from the 1000 Genome Project and only rare variants with a frequency of <0.01 will be analysed further (rare variants). Vitiligo and autoimmune disorders are individually common, but the co-occurrence of these disorders is not common. The variants with a frequency of <0.01 are deemed to be rare, and since the phenotype is relatively rare within the population, the variant is also likely to be rare. Of the prioritized variants, the variants (in the selected genes) that are common amongst participants with vitiligo and other autoimmune disorders will be compared to the variants identified in unaffected family members (Family 1: probands unaffected mother, and her cousin, Family 2: proband and his parents).

4.3.3. Bioinformatics and filtering

Modern and user-friendly bioinformatics tools for analysis and interpretation of genomics data become essential during the analysis of sequenced data (Karabayev et al., 2021). Exome sequencing will produce large raw data in the form of a BAM files, index files and processed user-ready file in the form of variant call files (VCF). A VCF is the output of a bioinformatics pipeline that stores information on variants present in the tested individual with respect to the reference genome (build 37). A VCF will be uploaded in Ensembl's Variant Effect Predictor (VEP) and will annotate the variants according to the type of consequence, further shown in figure 3 below. The Integrative genomics viewer (IGV) will then be used to visualise reads to confirm the variants and assess the sequencing coverageand d

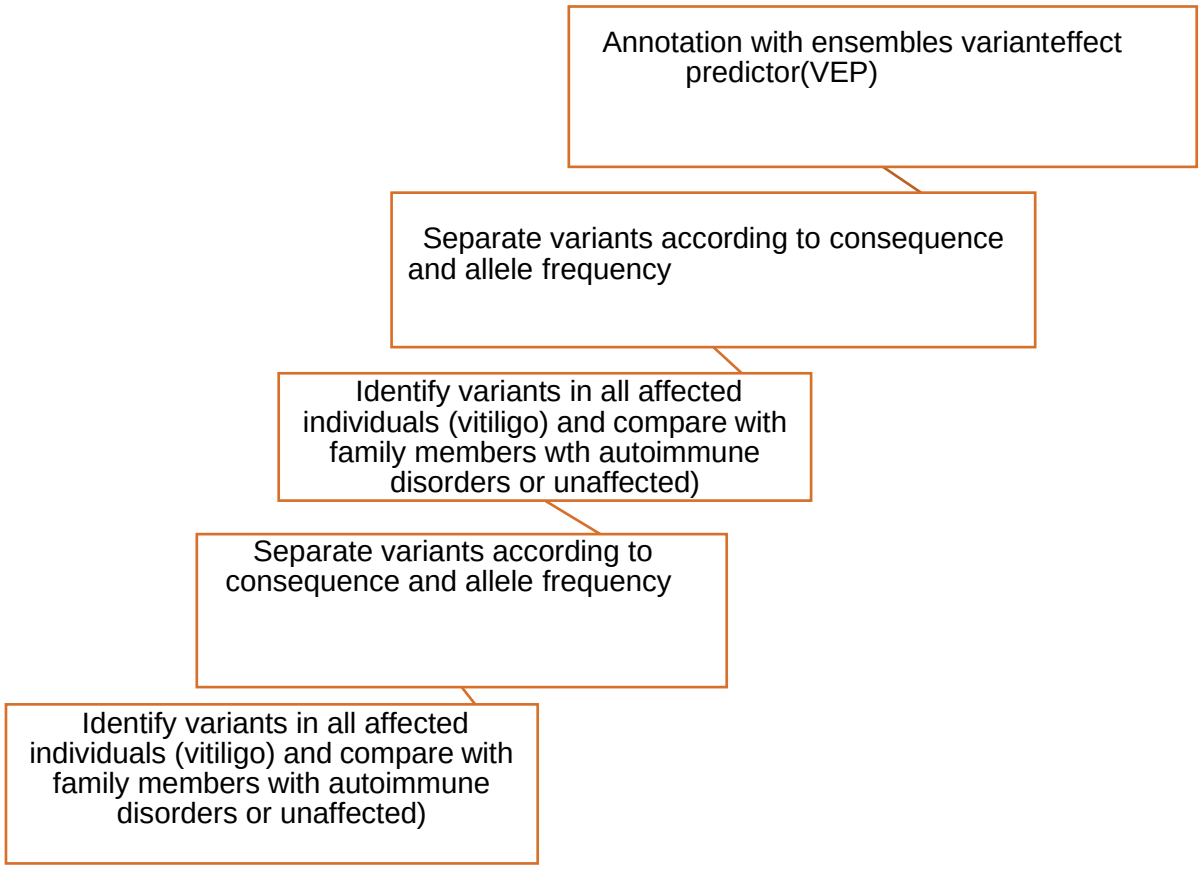


Figure 3: Flow chart showing data analysis strategy for exome sequencing dataanalysis

5. Genotyping the *SMOC2* rs13208776 SNP

SMOC2 rs13208776 was significantly associated with vitiligo in a large Romanian family withvitiligo and autoimmune disorders (Birlea, et al., 2010). However, rs1320876 was not significantly associated with vitiligo or autoimmune disorders in a Jordanian cohort with vitiligo and other autoimmune disorders (Alkhateeb, et al., 2010). Although the association ofthis SNP was found in a founder population, the families that will investigated in this study have a diseases background where vitiligo and other autoimmune disorders are found in the families across multiple generations. Although we have access to two families with vitiligo (multi generations) and at least one autoimmune disorder, we cannot perform an associationstudy. It would be informative to determine whether this SNP is present in these families thathave a similar disorder pattern to those seen in the families used for the Romanian and Jordanian association studies. The SNP will be genotyped using the TaqMan assay.

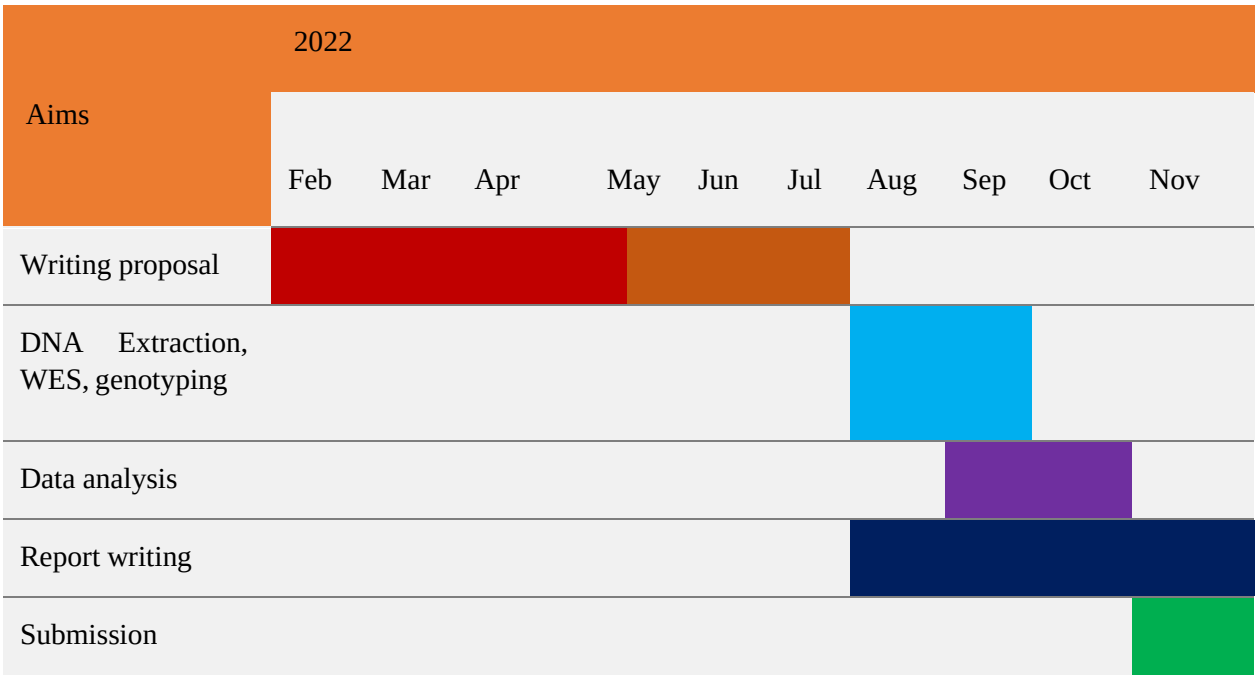
TaqMan genotyping assays are used to amplify and detect specific alleles in genomic DNA (gDNA). The TaqMan genotyping assay is a PCR-based assay for genotyping single nucleotide polymorphisms (SNPs). The region flanking the SNP is amplified in the presenceof two allele-specific fluorescent probes. The probes do not emit fluoresce in solution because of a quencher at the 3′ ends that inhibit flourescence emission while the quencher

is in close proximity to the flouropore. The presence of two probes allows the detection of both alleles in a single tube. Genotyping will be performed on the Quantstudio 3 (in-house)using a pre-designed Thermofisher rs13208776 TaqMan assay.

6. Timing

An estimation of how much time will be allocated throughout the proposed research project toachieve the aim and objectives of the study is shown in Table 3.

Table 3: Gantt chart representing how time will be allocated throughout the study



7. Funding

This research project will be funded by the NRF Thuthuka grant of Dr. T. Ngcungcu. All reagents required for this study have already been purchased except for the Taqman SNPgenotyping reagents. Table 4. indicates the budget for this study.

Table 4: Budget for the study

Description	Price
Taqman SNP genotyping Assay and Mastermix	R17,952.77
General consumables	R2,000.00
Ion Ampliseq Exome RDY kit	R32,048.00
Ion 540 chip kit(each)	R24,780.00
Ion 540 KIT-chef 8RXNS	R13,780.00
Total amount	**R90560.77**

8. Ethics

This research project forms part of the umbrella project "The genetic of aetiology of vitiligo in Africans" (M200504). This is a Sub study and Ethics application has been approved by the University of Witwatersrand Human Research Ethics Committee (HREC, subject to protocolapproval). Protocol no is M220389. Both certificates are attached in appendix B.

9. Limitations

- The sample size is small. Should a mutation be identified, we will not know if the mutation is limited to the study samples or if the variant is found in other individualswith similar phenotypes.
- Noncoding variants will not be sequenced because of WES.
- Only five non-MHC will be analysed, if some mutations are in the MHC or other non-MHC genes, they will be missed.
- Due to time constraints, potential causal variants will not be validated with Sanger sequencing, which is commonly done to validate next generation sequencing findings.

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11 Appendices

Appendix A: Complete pedigree of family 1

Numbering of individuals in figure 1 is not the same as numbering in appendix A below, as figure 1 is a zoomed-in part of the complete pedigree of family 1 as shown with appendix A. However, individuals with a WIT ID in appendix A are the same as those with a VIT ID in figure1.

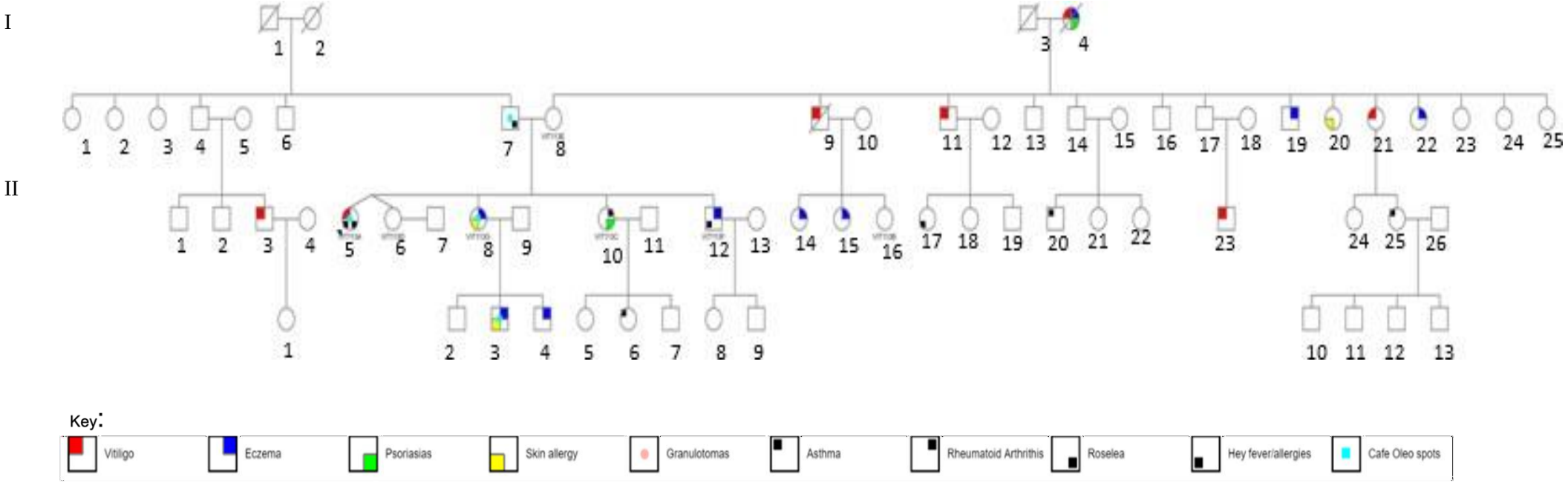

Figure A1: Pedigree of family 1. The probands (VIT113A) maternal side shows multigenerational tracking of vitiligo and various autoimmunediseases. The individuals with an VIT ID will be used in this study.

Appendix B: Ethics certificates

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr T Ngcungcu

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200504

NAME: (Principal Investigator)	Dr T Ngcungcu
DEPARTMENT:	School of Pathology Department of Human Genetics Medical School University
PROJECT TITLE:	The genetic aetiology of vitiligo in Africans
DATE CONSIDERED:	2020/05/29
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Not applicable
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/08/25

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **May** and will therefore reports and re-certification will be due early in the month of **May** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

25/08/2020
Date

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms K Rabinda

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220389

NAME: (Principal Investigator)	Ms K Rabinda
DEPARTMENT:	School of Pathology Division of Human Genetics Medical School University
PROJECT TITLE:	*Exome sequencing of family with vitiligo and other autoimmune disorders*
DATE CONSIDERED:	Ad hoc
DECISION:	Approved conditionally
CONDITIONS:	Sub-study under M200504 The HREC requires evidence of Faculty protocol approval
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Dr T Ngcungcu
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/03/30

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Philip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **March** and therefore reports and re-certification will be due in the month of **March** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

31/03/2022
Date

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms K Rabinda

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220389

NAME: (Principal Investigator)	Ms K Rabinda
DEPARTMENT:	School of Pathology Division of Human Genetics Medical School University
PROJECT TITLE:	*Exome sequencing of family with vitiligo and other autoimmune disorders*
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M200504 Prior condition (Faculty protocol approval) satisfied on 2022/09/01
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Dr T Ngcungcu
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/03/30

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **March** and therefore reports and re-certification will be due in the month of **March** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

11/11/2022

Date

Appendix C

Turn-it-in Report

MSc(Genomic Medicine)_1132300_Research_Report.pdf

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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Appendix D

Authors Guidelines

Article Title

First Author[1], Second Author[2*], Third Author[1,2]

[1]Laboratory X, Institute X, Department X, Organization X, City X, State XX (only USA, Canada and Australia), Country
[2]Laboratory X, Institute X, Department X, Organization X, City X, State XX (only USA, Canada and Australia), Country

*** Correspondence:**
Corresponding Author
email@uni.edu

Keywords: keyword$_1$, keyword$_2$, keyword$_3$, keyword$_4$, keyword$_5$. (Min.5-Max. 8)

Abstract

For full guidelines please refer to Author Guidelines

As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. References should not be cited in the abstract. Leave the Abstract empty if your article does not require one – please see the "Article types" on every Frontiers journal page for full details.

1 Introduction

For **Original Research Articles, Clinical Trial Articles**, and **Technology Reports** the introduction should be succinct, with no subheadings. For **Case Reports** the Introduction should include symptoms at presentation, physical exams and lab results.

2 Article types

For requirements for a specific article type please refer to the Article Types on any Frontiers journal page. Please also refer to Author Guidelines for further information on how to organize your manuscript in the required sections or their equivalents for your field[1].

[1] For Original Research articles, please note that the Material and Methods section can be placed in any of the following ways: before Results, before Discussion or after Discussion.

3 Manuscript Formatting

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You may insert up to 5 heading levels into your manuscript as can be seen in “Styles” tab of this template. These formatting styles are meant as a guide, as long as the heading levels are clear, Frontiers style will be applied during typesetting.

3.2 Equations

The equations should be inserted in editable format from the equation editor.

$$f(x) = a_0 + \sum_{n=1}^{\infty} \left(a_n \cos \frac{n\pi x}{L} + b_n \sin \frac{n\pi x}{L}\right)$$

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Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript. Figures will then be automatically embedded at the bottom of the submitted manuscript. Kindly ensure that each table and figure is mentioned in the text and in numerical order. Figures must be of sufficient resolution for publication. Figures which are not according to the guidelines will cause substantial delay during the production process.
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Tables should be inserted at the end of the manuscript. Tables must be provided in an editable format e.g., Word, Excel. Tables provided as jpeg/tiff files will **not be accepted**. Please note that very large tables (covering several pages) cannot be included in the final PDF for reasons of space. **These tables will be published as Supplementary Material on the online article**

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To take part in the Resource Identification Initiative, please use the corresponding catalog number and RRID in your current manuscript. For more information about the project and for steps on how to search for an RRID, please click here.

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Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords with the following format:

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6 Conflict of Interest

All financial, commercial or other relationships that might be perceived by the academic community as representing a potential conflict of interest must be disclosed. If no such relationship exists, authors will be asked to confirm the following statement:
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

The Author Contributions section is mandatory for all articles, including articles by sole authors. If an appropriate statement is not provided on submission, a standard one will be inserted during the production process. The Author Contributions statement must describe the contributions of individual authors referred to by their initials and, in doing so, all authors

agree to be accountable for the content of the work. Please see here for full authorship criteria.

8 Funding

Details of all funding sources should be provided, including grant numbers if applicable. Please ensure to add all necessary funding information, as after publication this is no longer possible.

9 Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

10 Reference styles

The following formatting styles are meant as a guide, as long as the full citation is complete and clear, Frontiers referencing style will be applied during typesetting.

10.1 Science, Engineering and Humanities and Social Sciences references

For articles submitted in the domains of Science, Engineering or Humanities and Social Sciences please apply Author-Year system for in-text citations. For Humanities and Social Sciences articles please include page numbers in the in-text citations

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11 Supplementary Material

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12 Data Availability Statement

The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF REPOSITORY] [LINK]. Please see the "Availability of data" section of Materials and data policies in the Author guidelines for more details.