Genetic Investigation of South Africans with the Noonan Syndrome Phenotype using Targeted Next Generation Sequencing

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DECLARATION

I, ***Cedrik Ngongang Tekendo***, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

Introduction

Noonan Syndrome (NS) is an autosomal dominant multisystem disorder, characterised by short stature, distinctive facial dysmorphism, cardiovascular abnormalities and developmental delay. Its estimated incidence is 1:1000 to 1:2500 live births. NS is caused by germline mutationsin more than ten genes encoding proteins integral to the Ras/MAPK signaling pathway. Pathogenic variants in these genes account for 70-80% of NS cases. The clinical diagnosis of NS can be challenging in some cases, even when performed by experienced clinicians. The introduction of Next Generation Sequencing (NGS) technology in clinical practice in the Western world has tremendously facilitated the molecular diagnosis of RASopathies. Molecular testing for NS is not yet available in South Africa, nor has any study investigating NS from clinical and molecular perspectives been conducted in South Africans.

Aim

The aim of this study was to investigate selected genes within a group of paediatric and adult patients with a clinical diagnosis of NS.

Methods

This study was a cross-sectional descriptive study, including twenty-six familial and isolated NS patients recruited in Cape Town in the period January 2015-January 2017. Thorough phenotyping of each patient according to the international diagnostic criteria for NS was followed by targeted NGS, performed on leucocyte DNA samples from sixteen unrelated patients out of the twenty-six included. Sequencing involved all the exons and intron-exon boundaries of a predesigned panel of 14 genes, including A2ML1, BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, RIT1, SHOC2, SOS1 and SPRED1.

Results

Of the 26 patients included, 50% had a family history suggestive of NS.The median age at diagnosis was4.5 years (range: 1month-51years). Individuals of mixed-race ancestry were most represented (53.8%), followed by black Africans (30.8%). The clinical features identified were consistent with those reported in other populations.

Compared to other series, our cohort revealed a lower frequency of Pulmonary Valve Stenosis (34.6%) and a less severe developmental phenotype.

Variants predicted pathogenic were detected in 7(43.7%) DNA samples out of the 16 analysed. The genes involved were *CBL* in three cases (42.8%), *PTPN11*and *MAP2K1*in two cases (28.6%, for each gene). Surprisingly,the proportion of *CBL* variants was relatively high compared to those in the literature. Genotype-phenotype correlations showed that clinical features of NS were more typical in patients with pathogenic variants in *MAP2K1*, and less in those with variants in *CBL*.

Conclusion

This is the first clinical and molecular study in South Africanswith the NS phenotype. The phenotype of affected individuals with NS in South Africa is globally similar to that reported in the literature. Therefore, the use of international diagnostic criteria can effectively enable the clinical diagnosis of NS in most South African patients.

These preliminary data suggest that the distribution of pathogenic variants in NS genes in South Africans may be different from that reported in other populations. Finally, this study demonstrates that Targeted NGS can be successfully applied to the molecular diagnosis of NS and related conditions in South Africa, and should be implemented in clinical practice.

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LIST OF ABBREVIATIONS

A2ML1:alpha-2-macroglobulin like 1

aPTT: activated partial prothromboplastin time

BRAF:B-Raf proto-oncogene, serine/threonine kinase

bp: base pair

CBL:Cbl proto-oncogene

CFC: Cardio-facio-cutaneous Syndrome

CHD: Congenital heart defect

CS: Costello Syndrome

DNA: Deoxyribonucleic acid

ECG: Electrocardiogram

EDTA: Ethylenediaminetetraacetic acid

GSH: Groote Schuur Hospital

GTP: Guanosine triphosphate

HCM: Hypertrophic cardiomyopathy

Hg19: Human genomes build 19

HREC: Human Research EthicsCommittee

KRAS:KRAS proto-oncogene, GTPase

MAF: Minor allele frequency

MAPK: Mitogen-activated protein kinase

MAP2K1:Mitogen-activated protein kinase kinase 1

MAP2K2: Mitogen-activated protein kinase kinase 2

MEK1:MAP kinase/ ERK kinase 1

MPS: massive parallel sequencing

MWM: Molecular weight marker

NGS: next generation sequencing

NHLS: National Health Laboratory Service

NRAS:NRAS proto-oncogene, GTPase

NS: Noonan Syndrome

PCR: polymerase chain reaction

PDA: patent ductus arteriosus

PS: pulmonary valve stenosis

PT: partial prothrombin time

PTPN11: protein tyrosine phosphatase, non-receptor type 11

RAF1:Raf-1 proto-oncogene, serine/threonine kinase

Ras: Retrovirus-associated DNA sequences

RASA2: RAS p21 proteinactivator 2

RCWMCH: Red CrossWar Memorial Children'sHospital

RIT1:Ras like without CAAX 1

RRAS:Related RAS viral (r-ras) oncogene homolog

SHOC2:Leucine rich repeat (LRR) scaffold protein in Homo sapiens

SOS1: Son of sevenless homolog 1

SOS2: Son of sevenless homolog 2

UCT: University of Cape Town

VCF: Variant call format

WES: whole exome sequencing

WGS: whole genome sequencing

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 THE RASOPATHIES

The RASopathies are a well-defined group of clinically and genetically related developmental disorders, caused by germline pathogenic variants in genes playing a role in the Ras/Mitogen-Activated Protein Kinase (MAPK) signalling pathway (Tidyman & Rauen 2009). This well-studied signal transduction pathway is critical for normal mammalian development, with the control of several vital cellular functions including cell cycle, cell growth, differentiation and survival (Yoon & Seger 2006; Rauen 2013). While each RASopathy has a distinctive phenotype, RASopathies share several clinical characteristics because of their similar effect on the dysregulation of the Ras/MAPK pathway. This group of genetic disorders includes: Neurofibromatosis Type 1 (NF-1), Noonan Syndrome (NS), Costello Syndrome (CS), Cardiofaciocutaneous Syndrome (CFC), Noonan Syndrome with multiple lentigines previously known as LEOPARD syndrome (NSML), Legius Syndrome (LS) and Capillary Malformation-Arteriovenous Malformation Syndrome (CM-AVM) (Figure 1).

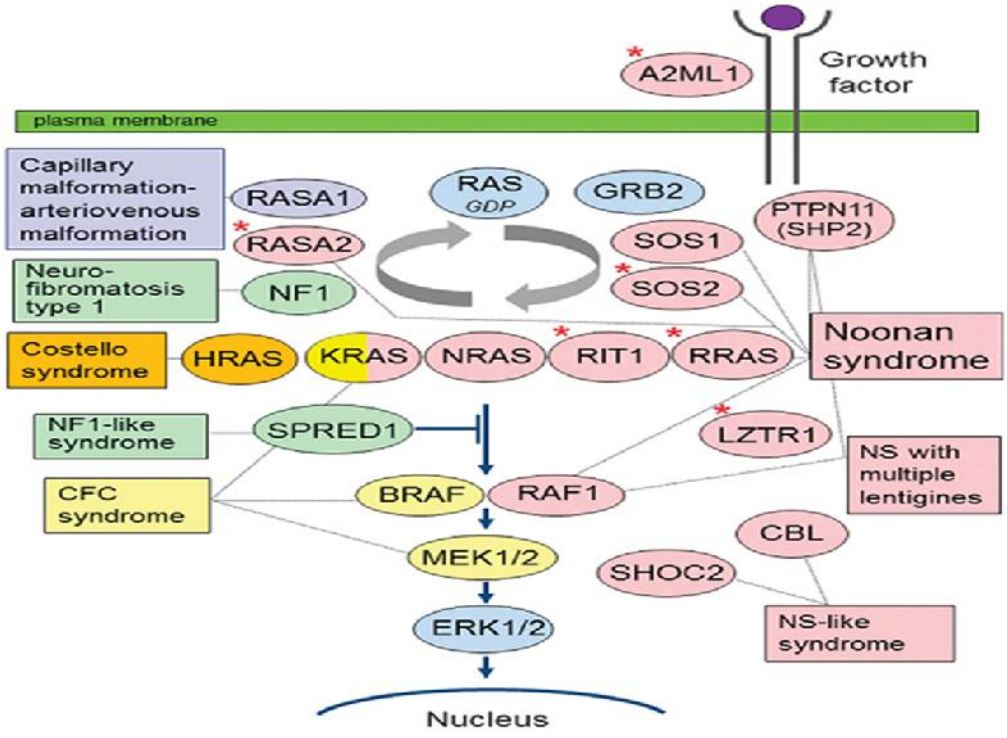

Figure 1: The Ras/MAPK signaling pathway (*Adapted from*Aoki et al. 2016).
This figure illustrates the relationship between known RASopathies and their associated genes in the Ras/MAPK signalling pathway. The star (*) indicates putative causative genes recently reported.

1.2 NOONAN SYNDROME

Noonan Syndrome (NS) is likely the second most common RASopathy after Neurofibromatosis Type 1, with an estimated incidence of 1:1000 to 1:2500 live births (Mendez & Opitz 1985). It is caused by germline mutations in several genes known as either components or regulators of the Ras/MAPK signalling pathway (Tidyman & Rauen 2009). NS is transmitted following autosomal dominant pattern of inheritance owing to family clustering, with 30-75% of patients having an affected parent (Allanson & Roberts 2001).

1.2.1 CLINICAL PRESENTATION

Individuals affected by NS present with multisystem involvement, including short stature, distinctive facial dysmorphism and a wide range of cardiovascular and other abnormalities (Figure 2 and Tables 1, 2&3). It is believed that many physical features of NS are caused by lymphatic dysfunction or obstruction during development (Allanson 2007).

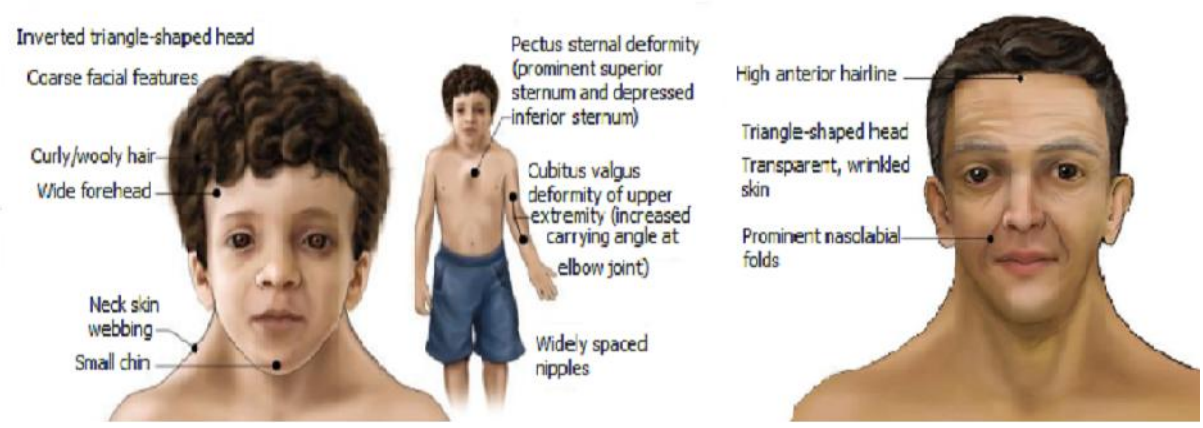

Figure 2: **Child/adolescent and adult with Noonan Syndrome** (*adapted from Bhambhani & Muenke 2014)*. This figure provides a graphic representation of the dysmorphic features found in NS.

The condition is clinically heterogeneous with variable expressivity, even within the same affected family. Some physical features associated with NS, especially the facial dysmorphism, alter with age and can considerably vary among different age groups (Allanson et al. 1985; Roberts et al. 2013).

Table 1: **Typical facial features of NS by age group** (*Adapted from*Roberts et al. 2013) and Romano et al. 2010)

The table below presents and compares the facial features of NS in four age groups.

	Forehead, face, hair	Eyes	Ears	Nose	Mouth	Neck
Newborn	Tall forehead, low posterior hairline	Hypertelorism, downslanting palpebral fissures, epicanthic folds	..	Short and broad, de-pressed root, upturned tip	Deeply grooved philtrum, high & wide peaks of the vermilion, micrognathia	Excessive nuchal skin
Infancy **(2-12 months)**	Macrocephaly Tall & prominent forehead	Hypertelorism, ptosis, or thick-hooded eyelids	..	Short, wide; depressed nasal root	..	..
Childhood **(1-12 years)**	Coarse features elongated face	..	..	..	..	..
Adolescence **(12-18 years)**	Myopathic facies	..	..	High, thin nasal bridge	..	Webbing
Adulthood **(> 18 years)**	Distinguishing facial features are subtle, skin appears thin and transparent	..	..	Prominent nasolabial fold	..	..
All ages	..	Blue-green irises, arched & diamond-shaped eyebrows	Low-set, thick helices	..	..	..

Table 2: **Cardiovascular features of NS**

Congenital heart diseases (CHDs) are present in 50-80% of individuals with NS. The most common is Pulmonary Valve Stenosis, usually with a dysplastic valve. In NS, Hypertrophic Cardiomyopathy (HCM) can occur in the prenatal or postnatal period.

	Features	Percent (%)	References
Congenital heart defects (CHD)	Pulmonary valve stenosis Dysplastic pulmonary valve Secundum atrial septal defects Ventricular septal defects Others less common (Aortic coarctation, mitral valve defects, Tetralogy of fallot, Patent ductus arteriosus)	(50-60) (25-33) (10-32) (12)	(Colquitt & Noonan 2013; Prendiville et al. 2014; Van der Burgt 2007)
Cardiomyopathy	Hypertrophic cardiomyopathy	(14-30)	(Raaijmakers et al. 2008; Hickey et al. 2011)
Electrocardiographic abnormalities	Left axis deviation Negative aVF Abnormal Q wave	(90)	(Sharland et al. 1992; Raaijmakers et al. 2008)

Table 3: **Other clinical features in NS**

This table provides a summary of the clinical manifestations of NS reported in clinical studies on NS in the literature:

Anomalies	Features	Percent (%)	References
Growth	Short stature	50-70	(Mendez HM 1985; Sharland et al. 1992)
Musculo-skeletal	Chest o Pectus carinatum superiorly o Pectus excavatum inferiorly Spine o Scoliosis o Other vertebral anomalies Limbs o Cubitus valgus o Talipes equinovarus Joints o Contracture o Radio-ulnar synostosis o Cervical spine fusion o Hyper extensibility	(70 –95) 10 – 15 50 10 – 15 4 2 2 50	(Sharland et al. 1992; Shaw et al. 2007; Van der Burgt 2007)
Ophthalmology	Strabismus Anterior segment changes Refractive errors Amblyopia Fundal abnormalities Nystagmus	(90-95) 48 – 63 63 61 33 20 10	(Sharland et al. 1992; Naficy et al. 1997)
Central Nervous System	Arnold-Chiari malformation type I Hydrocephalus		(Holder-Espinasse & Winter 2003)
Haematology & Oncology	Bleeding diathesis o Easy bruising o Intrinsic pathway abnormalities (factors VIII, XI and XII deficiencies most frequently) o Thrombocytopenia o Platelet function abnormalities Transient Myeloproliferative disorder Juvenile myelomonocytic Leukaemia Acute myeloid Leukaemia Acute lymphoblastic leukaemia Solid tumours o Rhabdomyosarcoma o Neuroblastoma	(30-65) 46 - 58 50 –90	(Sharland et al. 1992; Artoni et al. 2014; Strullu et al. 2014; Denayer et al. 2010)

Gastro-intestinal	Feeding difficulties: o Poor suck with prolonged feeding time o Very poor suck and slow feeding with recurrent vomiting o Severe feeding problems that require tube-feeding for ≥2 weeks Hepatomegaly in infancy	(76) 15 38 24 (26 -51)	(Romano et al. 2010; Sharland et al. 1992)
Genitourinary	Undescended testes Renal abnormalities Sertoli cell dysfunction in males	60 - 80 10-11	(Sharland et al. 1992; Marcus et al. 2008)
Oral and Dental	Dental malocclusion High arched palate Articulation difficulties Micrognathia Giant cell lesions	50 - 67 55-100 72 33 – 43	(KUMAR et al. 2014; Neumann et al. 2009)
Auditory	Sensorineural hearing loss Hearing loss secondary to otitis media Conductive hearing loss	50 15 - 40	(Qiu et al. 1998; Van der Burgt 2007)
Neurology, Education and Behaviour	Mild intellectual disability Recurrent seizures Learning difficulties Irritability Poor coordination	15 – 35 13 10 – 40	(Van der Burgt 2007; Shaw et al. 2007)
Developmental milestones	Mild motor delay o Sitting alone: 10 months o Walking: 21 months Speech delay o Speaking 2-word sentences: 31-32 months		(Sharland et al. 1992)
Lymphatic	Lymphedema (most common) Lymphangiectasis Chylous effusion Others	(~20)	(Witt et al. 1987; Romano et al. 2010)
Dermatology	Pigmented naevi Café au lait spots Keratosis pilaris atrophicans faciei Thick curly hair Thin sparse hair	25 10 14 33 10	(Sharland et al. 1992)

1.2.2 MOLECULAR GENETICS

At the molecular level, NS is highly heterogeneous with heterozygous germline pathogenic variants reported in more than ten genes (Aoki et al. 2016), all of them encoding proteins which are components or regulators of the Ras/MAPK signalling pathway. NS-causing genes include genes that encode proteins of the Ras family of GTPases (*KRAS, NRAS, RIT1*, and *RRAS*), modulators of Ras function (*PTPN11, SOS1, SOS2, A2ML1, CBL, RASA2*, and *SHOC2)* and downstream signal transducers (*RAF1, BRAF and MEK1*or MAP2K1) (Tartaglia et al. 2001;Roberts et al. 2007; Aoki et al. 2013;Flex et al. 2014;Vissers et al. 2015; Yamamoto et al. 2015; Cordeddu et al. 2015). Mutations associated with NS in these genes result in gain-of-function within the Ras/MAPK signalling pathway, and account for approximately 80% of all NS cases (Cordeddu et al. 2015). Missense mutations in *PTPN11* alone are found in about 50% of affected individuals (Tartaglia & Gelb 2005), while *SOS1* has been reported to be the second most mutated gene, accounting for 10-20% of *PTPN11*-negative patients (Roberts et al. 2007).

1.2.3 CLINICAL AND MOLECULAR DIAGNOSIS

1.2.3.1 CLINICAL DIAGNOSIS

The clinical diagnosis of NS is made through recognition of a combination of key physical features and cardiovascular abnormalities, with or without a positive family history (Romano et al. 2010). A number of clinical scoring systems have been proposed to assist in the diagnostic process of NS, including a comprehensive diagnostic scoring system combining minor and major features established by Van der Burgt and collaborators in 1994(Table 4) (Van der Burgt 2007). The clinical diagnosis of NS can be more difficult in adulthood, as many adults present with subtle clinical features. Most adults are diagnosed for the first time after the birth of an affected child (Shaw et al. 2007). Furthermore, overlapping features with other RASopathies, especially Cardiofaciocutaneous Syndrome and Costello Syndrome, can make the clinical diagnosis of NS very challenging in some cases.

Table 4: Diagnostic scoring system for NS(Van der Burgt 2007)

This table provides the diagnostic scoring system for NS elaborated by Van der Burgt in 1994.

Definitive NS: Criterion 1A + one of 2A-6A or 1A + two of 2B-6B

Criterion 1B + two of 2A-6A or 1B + three of 2B-6B

Feature	A = Major	B = Minor
1 Facial	Typical face dysmorphology (Allanson. 1987)	Suggestive face dysmorphology
2 Cardiac	Pulmonary valve stenosis, HCM and/or ECG typical of NS	Other defects
3 Height	<3 centile	<10 centile
4 Chest wall	Pectus carinatum/excavatum	Broad thorax
5 Family history	First degree relative with definite NS	First degree relative with suggestive NS
6 Other	Developmental delay, cryptorchidism **and** lymphatic dysplasia	One of developmental delays, cryptorchidism **or** lymphatic dysplasia

1.2.3.2 MOLECULAR DIAGNOSIS

The molecular diagnosis of NS involves the testing of specific genes known to be associated with the disorder. Different testing approaches can be used in clinical settings:

a. Single gene analysis

This approach involves the use of Sanger sequencing to test for specific variants in a selected gene (Tartaglia et al. 2001). This traditional method has been extensively used for the molecular diagnosis of NS, and remains the gold-standard DNA sequencing technique for confirmation of sequence variants(Tartaglia et al. 2001; Roberts et al. 2007). Due to high genetic heterogeneity, the typical diagnostic testing protocol for NS requires a multi-step approach, using PCR and Sanger sequencing. The selection of which gene or genes to investigate as a first diagnostic step is based on the frequency of association with the condition and the relationship with a particular phenotype (Lepri et al. 2014). The downside of using Sanger sequencing is that only one PCR fragment can be sequenced at a time, making this method laborious, time consuming and expensive.

b. Multigene analysis

This approach involves the use of massively parallel sequencing (MPS), also known as next generation sequencing (NGS), which allows for the simultaneous testing of multiple genes (Teer & Mullikin 2010). In clinical settings, this can be achieved using at least two methods:

- Whole exome sequencing

Whole exome sequencing (WES) covers the entire protein-coding region of the genome and as such, is capable of detecting known disease-causing variants as well as new mutations leading to the discovery of novel gene–disease associations. While highly effective, WES presents a number of difficulties in a clinical setting, mostly relating to the challenges of high throughput data analysis, the interpretation and validation of findings, and the possibility of incidental findings(Rehm et al. 2013).Furthermore, the non-uniformity of the depth of coverage for an exome may lower its analytical sensitivity, which may result in a lack of sufficient coverage in genes/regions of interest (Rehm et al. 2013).

- Disease-targeted gene panel

This method is used to investigate well-known disease-associated genes. Targeted gene sequencing allows for the testing of specific genes of interest at the same time, as opposed to the whole genome or exome (Rehm et al. 2013). This targeted approach allows greater depth of coverage, with higher analytical sensitivity and specificity than WES. Moreover, because only well-known disease-associated genes are sequenced, data analysis and interpretation in a clinical context are less complex. Targeting fewer genes also allows for a higher sample throughput in a clinical laboratory, compared to WES (Rehm et al. 2013). A disease-targeted gene panel is currently seen as the most effective approach in a clinical setting, and has been efficiently used for the molecular diagnosis of NS (Lepri et al. 2014).

1.2.4 MANAGEMENT

Comprehensive management guidelines for NS have been developed by several consortia, including an American consortium (Romano et al. 2010), a European consortium (Noonan Syndrome Guideline Development Group 2010) and other authors (Allanson 2007;Roberts et al. 2013).

1.2.4.1 EVALUATION FOLLOWING INITIAL DIAGNOSIS

The following evaluations are recommended in newly diagnosed individuals with NS:

- **General**: complete clinical and neurological examination, medical genetics consultation, molecular genetic testing and eventually, genetic counselling
- **Growth and feeding**: plotting of growth parameters on NS special growth charts
- **Development**: developmental evaluation by a multidisciplinary team
- **Cardiovascular**: cardiovascular examination by a cardiologist, electrocardiogram (ECG) and echocardiogram
- **Haematological**: coagulation screen including complete blood count with differential, prothrombin time (PT) or activated partial prothromboplastin time (aPTT)
- **Dental**: dental assessment between 1-2 years of age
- **Audiology**: baseline hearing assessment
- **Ophthalmology**: baseline ophthalmologic examination
- **Kidney**: baseline renal ultrasound
- **Skeletal**: clinical assessment of spine and rib cage with radiography, if indicated

1.2.4.2 LONG TERM FOLLOW-UP

The long term follow-up plan recommended in affected individuals is as follows:

- **General**: yearly complete clinical and neurological examination; return to medical geneticist if negative genotype; genetic counselling when adolescent or young adult
- **Growth and feeding**: plotting of growth parameters on NS special growth charts three times per year until 3 years old and yearly thereafter
- **Development**: developmental assessment yearly for children aged 5-18 years
- **Cardiovascular**: follow-up based on initial findings; if the heart is normal initially, repeat assessment every 5 years. Adults should not discontinue their cardiac evaluations even if initially normal in childhood or adolescence

- **Haematological**: repeat coagulation screen including complete blood count with differential, PT/aPTT after 6-12 months if the initial screen was done in infancy; pre-operatively, perform coagulation screen including complete blood count with differential, PT/aPTT and in consultation with haematologist: factor IX, XI, and XII concentrations, Von Willebrand factor, platelet function (bleeding time or platelet aggregation)
- **Audiology**: repeat hearing assessment if there is recurrent otitis or speech delay
- **Ophthalmology**: repeat ophthalmologic examination every two years or appropriately if initial evaluation is abnormal
- **Dental**: dental assessment between 1-2 years of age and yearly examination thereafter
- **Others**: for other organ-systems, follow-up or referral as indicated, depending on the initial evaluation.

1.2.4.3 TREATMENT OF MANIFESTATIONS

The treatment of NS-associated complications does not generally differ from standard treatments in the general population. Aspirin therapy should be avoided in affected individuals with a bleeding diathesis. (Allanson 2007; Noonan Syndrome Guideline Development Group 2010; Romano et al. 2010; Roberts et al. 2013).

1.2.5 GENETIC COUNSELLING

NS is inherited in an autosomal dominant fashion (Sharland et al. 1992).

1.2.5.1 RISK TO FAMILY MEMBERS

- **Parents**: approximately 30-75% of individuals newly diagnosed with NS have an affected parent (Allanson & Roberts 2001). In the remaining cases, the condition results from a *de novo* pathogenic variant. Thorough assessment of apparently healthy parents of a newly diagnosed child is recommended (Allanson & Roberts 2001).

- **Siblings**: the risk to the siblings depends on the genetic status of the parents. The risk to the siblings is 50% if one of the parents has the pathogenic variant identified in the proband. If the pathogenic variants identified in the proband are not detected in the leucocytes DNA of otherwise healthy parents, the risk to the siblings is low (<1%), but greater than that of the general population because of the possibility of germline mosaicism (Yoon et al. 2013).
- **Offspring**: the risk to each child of an affected individual with NS is 50%.
- **Other family members**: the risk to other family members depends on the status of the parents. They may be at risk if a proband's parent is affected.

1.2.5.2 PRENATAL AND PREIMPLANTATION GENETIC DIAGNOSIS

Prenatal genetic diagnosis for pregnancies at increased risk for NS and preimplantation genetic diagnosis can be offered to families with a known pathogenic variant previously identified in an affected family member (Roberts et al. 2013).

1.3 RATIONALE OF THE STUDY

With an incidence of 1 in 1000-2500 live births, NS is a common genetic condition worldwide (Mendez HM 1985). Affected individuals can have a relatively normal lifespan, subject to an early molecular diagnosis and appropriate long term follow-up (Noonan Syndrome Guideline Development Group 2010; Romano Alicia et al. 2010; Roberts et al. 2013).

The clinical diagnosis of NS is straightforward in many cases, but overlapping clinical characteristics with a number of RASopathies, as well as the subtle clinical presentation in some age groups (such as newborns and adults) can make the diagnosis problematic (Roberts et al. 2013; Lepri et al. 2014). Many familial and sporadic paediatric and adult cases have been clinically identified in South Africa, but to the best of our knowledge, molecular diagnosis is currently not available locally. Determining the molecular diagnosis of our patients may not only allow confirmation of our clinical findings, but may also facilitate timely family interventions through cascade screening. Moreover, successful introduction of NGS technology in clinical practice in the western world (Rehm et al. 2013; Lepri et al. 2014), as well as efforts currently made by the South African health system to do the same, could facilitate the establishmentof comprehensive gene panel testing for NS in the country. To the best of our knowledge, no research investigating the genetic aetiology of NS in South African patients has been previously conducted. The present study aims to characterise a cohort of South African NS patients from a clinical and genetic perspective. This would provide preliminary insights into the mutation prevalence in local NS patients and possible specific genotype/phenotype correlations and founder effects. The use of targeted gene panel NGS ultimately aims at establishing a molecular diagnostic service for NS in South Africa.

1.4 AIM AND OBJECTIVES OF THE STUDY

1.4.1 AIM

The aim of this study was to investigate selected genes within a group of paediatric and adult patients with a clinical diagnosis of Noonan Syndrome.

1.4.2 OBJECTIVES

The objectives of this study were:

a. To identify, both retrospectively and prospectively, familial and sporadic cases of NS;

b. To undertake a thorough phenotyping of each selected proband;

c. To perform molecular analysis on a genetic sample from each proband;

d. To establish Genotype-Phenotype correlationsofNS within the study population.

CHAPTER 2

MATERIAL AND METHODS

2.1 STUDY DESIGN

The present study is a cross-sectional and descriptive study.

2.2 PATIENT DESCRIPTION

2.2.1 ETHICAL APPROVAL

Ethical approval was granted by the Human Research Ethics Committee of the University of Cape Town (HREC REF: 449/2016). All adult participants signed consent forms, while informed consent was given by the parents of participants younger than 18 years of age.

2.2.2 CHARACTERISTICS OF THE STUDY POPULATION

2.2.2.1 STUDY POPULATION

Consecutive patients attending paediatric and adult genetic clinics at the Red Cross War Memorial Children's Hospital (RCWMCH) and Groote Schuur Hospital (GSH) respectively,between January 2015 and January 2017.

2.2.2.2 NUMBER OF PARTICIPANTS

Twenty six (n= 26) participants were selected using convenience sampling.

2.2.2.3 INCLUSION CRITERIA

- Adult and paediatric patients of both genders meeting the Van der Burgt scoring system criteria for clinical diagnosis of NS
- Familial and simplex cases of NS
- Available for clinical examination, provision of informed consent for genetic testing

2.2.2.4 EXCLUSION CRITERIA

- Patients not consenting to participate in the study

2.2.3 RECRUITMENT AND ENROLMENT

The recruitment was both retrospective and prospective. Retrospectively, known families and individual patients from the clinical database of the Division of Human Genetics, University of Cape Town (UCT) with a clinical diagnosis of NS were

contacted by telephone and invited by the candidate, Dr Cedrik Ngongang, in his capacity as genetic registrar, to attend special consultations aimed at obtaining informed consent and data collection. Prospectively, every new patient meeting the inclusion criteria and attending our paediatric or adult clinics during the recruitment period was enrolled. Informed consent and data collection were done by the candidate in the presence of the study supervisoratRCWMCH and GSH.

2.2.4 DATA COLLECTION METHODS

2.2.4.1 PATIENT SELECTION PROCEDURE

- Retrospectively and prospectively, probands were selected based on the adopted diagnostic scoring system developed by Van der Burgt(Van der Burgt 2007). The clinical diagnosis of NS was made in the presence of typical facial features plus an additional major feature or two minor features. In the presence of suggestive facial features, patients had to have at least two major features or three additional minor features. Major characteristics included cardiac abnormalities (pulmonary valve stenosis, HCM and/or ECG typical of NS), height below the 3rd centile, chest deformities (pectus carinatum/excavatum),positive family history (first degree relative with definite NS)and the following: undescended testes in male subjects, developmental delayand lymphatic dysplasia. Minor characteristics included any other cardiac defects, height below the 10th centile, broad thorax, first degree relative with suggestive NS and one of the following: developmental delay, undescended testes or lymphatic dysplasia.

- Retrospective phase: the patient database of the Division of Human Genetics, UCT, was reviewed by the candidate. All patients seen at our clinic in the last two years with a clinical diagnosis of NS were filtered. Individual patients in the case of adults, and parents (or guardians) in the case of children, were contacted by telephone and requested to attend one of the special consultations that were organised for the purpose of the present study. Each proband's folder was revisited and a careful clinical assessment was repeated by the candidate, together with the study supervisor. A questionnaire was administered to selected patients who consented to the study.

- Prospective phase: every new patient attending the routine adult or paediatric genetic clinic during the recruitment period and meeting the clinical diagnostic criteria of NS was directly enrolled into the study, if the candidate and the supervisor were both present. Alternatively patients with suggestive features were invited to attend one of the special consultations organised for the purpose of the study.

2.2.4.2 PHENOTYPING

FAMILIES' PEDIGREE

Family history of NS was assessed using a three-generation pedigree for each selected proband. The pedigree was extended on the affected side of the family whenevernecessary. A special enquiry was made on cardiovascular abnormalities, developmental delay or learning difficulties, and physical features of NS such as short stature, webbed neck and pectus deformities of the chest.

ANTENATAL FEATURES

Antenatal history of each selected patient was carefully recorded whenever possible. Information was collected from the parents, guardians, hospital files or any other available family archive. A focus was made on maternal illnesses, exposure to teratogens,and ultrasound abnormalities including soft markers and major foetal structural defects.

NOONAN DYSMORPHOLOGY ASSESSMENT

An extensive phenotyping of the probands was performed, allowing documentation of full clinical characteristics, by the candidate with additional examination with a consultant medical geneticist from the Division of Human Genetics, Faculty of Health Sciences, UCT. Every organ system was assessed, with special focus on those reported to be frequently affected in NS(Van der Burgt 2007; Romano et al. 2010; Roberts et al. 2013). Photographs of some proband were taken with their consent. Recommended basic initial investigations in NS, including laboratory testing and imaging were reviewed and completed as needed for each selected proban

CARDIAC EVALUATION

Echocardiograms and ECGs were routinely obtained for each selected patient through formal cardiac assessmentsby specialist cardiologists, either at RCWMCH for paediatric patients or GSH for adult patients. Data was collected from hospital files for those who had had cardiac evaluation prior to their medical genetics assessment. Cardiac assessment was systematically requested for patients with unknown cardiac phenotype. While echocardiograms were evaluated for CHDs (e.g. Pulmonary Valve Stenosis, HCM),ECGs were evaluated for abnormalities known to be associated with NS (e.g. left axis deviation) (Appendix 1).

BIOLOGICAL INVESTIGATIONS

Investigation of bleeding diathesis was done for each selected patient. A coagulation screen, including completeblood count, PT and aPTT was obtained either from patient's hospital files or by new request.

2.3 MOLECULAR METHODS

Part of our cohort of NS patients was genotyped for the coding sequences of a panel of 14 genes, including *A2ML1, BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, RIT1, SHOC2, SOS1 and SPRED1.* Deletion/duplication analysis was not performed in this study.

2.3.1 SAMPLE COLLECTION AND DNA EXTRACTION

Peripheral blood (2-5ml) was collected from each selected proband at the recruitment sites, after informed consent. Genomic DNA was subsequently isolated from peripheral blood samples at the NHLS- Molecular Genetics Laboratory, GSH,following the manufacturer's instructions (Maxwell®16 Blood DNA purification kit, Promega, Madison, WI 53711, USA).

2.3.2 DNA QUALITY CONTROL

A total of seventeen (n=17) DNA samples were checked for their purity, concentration and integrity (see summary in Table 5).

a. DNA purity and quantification

The purity (260/280 ratio) and initial concentration of the DNA samples were assessed using the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and its associated software, following standard procedures.
DNA samples were subsequently diluted using SABAX water to 50ng/uL according to Nanodrop concentrations, except when otherwise indicated. After calibration of the Qubit® 3.0 fluorometerwith standards (Thermo Fisher Scientific), DNA sample concentrations were calculated using the Qubit® dsDNA HS (High Sensitivity) Assay (Thermo Fisher Scientific) according to the manufacturer's protocol.

b. DNA integrity

To assess the integrity of DNA samples following the process of DNA isolation which is subject tomechanical damage, agarose gel electrophoresis was performed (Figure 3). A 1% (w/v) agarose gel (1X Tris-borate-EDTA buffer) was prepared. Approximately 100ng of each DNA sample (based on Qubit values) in addition to 5uL loading buffer (40% (w/v) sucrose, 0.125% (w/v) bromophenol blue) was analysed. The GeneRuler 100bp Plus DNA Ladder was chosen as the molecular weight marker (MWM) (Thermo Fisher Scientific). Electrophoresiswas performed at 110V for 30 minutes.

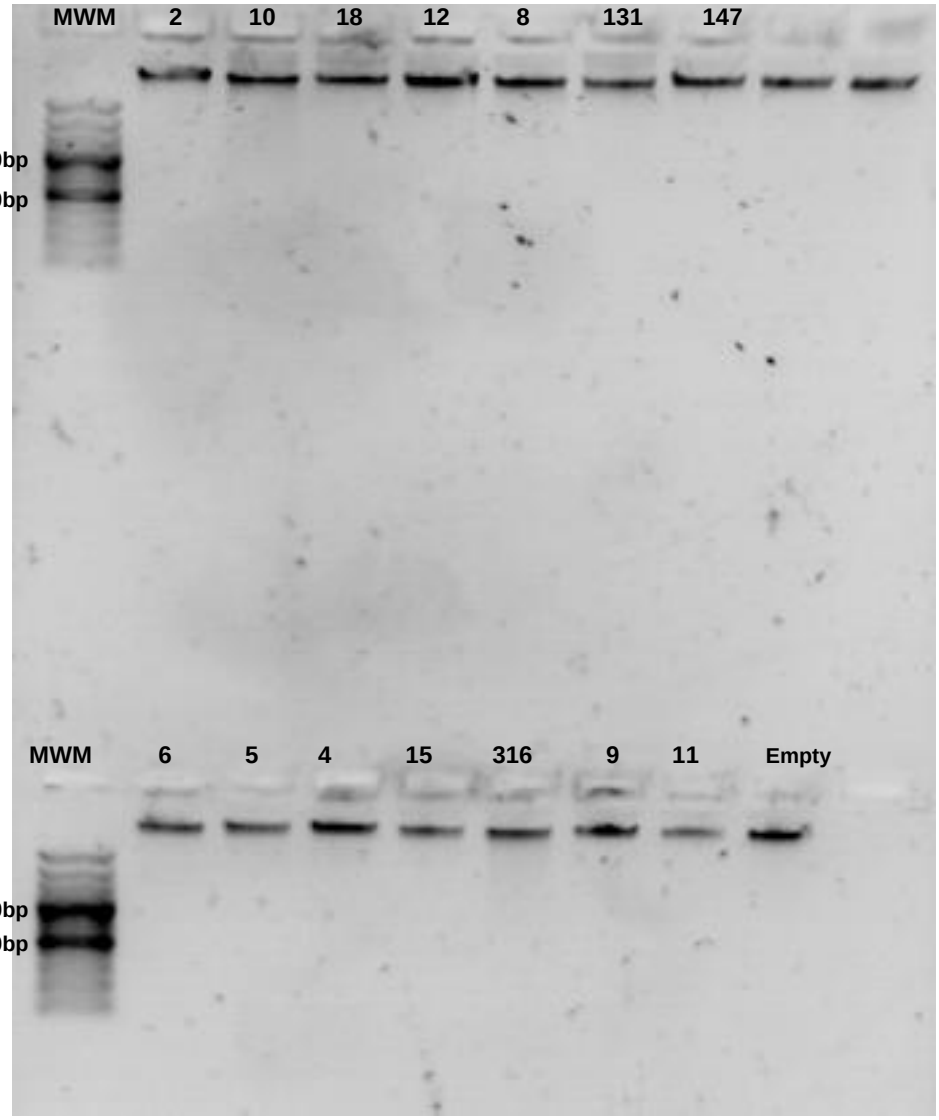

Figure 3: DNA integrity gel of NS patients

A1% (w/v) agarose gel electrophoresis product shows the resolution of each selected DNA sample. Lane numbers correspond to the respective patients.

- The gel at the top shows, from left to right: lane 1= GeneRuler 100bp Plus DNA Ladder molecular weight marker (**MWM**); lane 2= sample 02; lane 3 = sample 10; lane 4 = sample 18; lane 5 = sample 12; lane 6 = sample 08; lane 7 = sample 13; lane 8 = sample 01; lane 9 = sample 14 and lane 10 = sample 07.
- The gel at the bottom shows, from left to right: lane 1= **MWM**; lane 2 = sample 06; lane 3 = sample 05; lane 4 = sample 04; lane 5 = sample 15; lane 6 = sample 03; lane 7 = sample 16; lane 8 = sample 09; lane 9 = sample 11and lane 10 = empty

Table 5: **Summary of DNA Quality Control methods**

Sample ID	Nanodrop			Dilution			Qubit		Integrity gel	
	ng/uL	260/280	260/230	DNA (uL)	H2O (uL)	Comments	ng/uL	Comments	DNA (uL)	H2O (uL)
01	130.86	1.85	1.75	19	31		60		2	6
02	38.88	1.91	1.77	-	-	No dilution	33.6		3	5
03	55.4	1.87	20.76	-	-	No dilution	48.1		2	6
04	211.68	1.86	2.62	12	38		56		2	6
05	355.81	1.86	2.44	7	43		out of range	dilute 1:1 (25+25)	2	6
06	149.42	1.88	2.7	17	33		53		2	6
07	64.03	1.88	2.63	39	11		48.9		2	6
08	277.67	1.86	2.27	9	41		52		2	6
09	184.07	1.86	1.84	14	36		out of range	dilute 1:1 (25+25)	2	6
10	58.42	1.88	-10.33	43	7		36		3	5
11	112.87	1.94	1.8	22	28		36.2		3	5
12	777.11	1.89	2.2	3	47		out of range	dilute 1:1 (25+25)	2	6
13	300.47	1.86	2.09	8	42		58		2	6
14	109.96	1.89	4.12	23	27		out of range	dilute 1:1 (25+25)	2	6
15	116.03	1.85	2.25	22	28		44.2		2	6
16	98.29	1.88	3.24	13	12	final volume 25uL	50		2	6
17	1922.65	1.84	1.75	1	49		10.3		10	-

2.3.3 Ion TorrentNEXTGENERATION SEQUENCING

A total of sixteen (n=16) DNA samples were selected based on quality, purity and integrity, and genotyped using targeted NGS approach.

a. Primer Design

Online pre-designed primers for Ion AmpliSeq Noonan Research Panel (Nelen et al. 2014)were ordered using the Ion AmpliSeq Designer v5.1 software (Life Technologies, Carlsbad, CA). The primers amplify the coding sequences of the 14 genes mentioned before, which have been selected based on their role in NS, supported by recent published literature. The Ion AmpliSeq Noonan Research panel

(Nelen et al. 2014) is predicted to cover 100%, in 268 amplicons. Amplicons are split between two multiplex pools.

b. TaqMan RNase P ASSAY

Prior to library preparation, genomic DNA samples were quantified using TaqMan RNase P assay (ThermoFisher Scientific, USA), according to the manufacturer's protocol (https://tools.thermofisher.com/content/sfs/manuals/MAN0007732_SampleQuant_AmpliSeq_TaqManRNaseP_UB.pdf).

- Preparation of the standard curve with control DNA

Quantitative PCR (qPCR) was used to confirm the amplification efficiency of each genomic DNA sample and to obtain a more accurate estimation of concentration.The TaqMan RNase P assay (Thermo Fisher Scientific), which is recommended for NGS, was used. In order to generate a standard curve, a serial dilution was prepared with nuclease-free water for the control included in the kit, ranging from 5ng/uL to 0.15625ng/uL.

- DNA samples dilution, master mix preparation and real time PCR reaction

A 1:20 dilution of each genomic DNA sample with nuclease-free water was independently prepared, based on Qubit values. A PCR Master Mix was subsequently prepared for a 96-well plate and lastly, genomic DNA samples and controls were amplified in duplicate in 10uL reaction volumes according to the manufacturer's protocol, using a CFX96 (Bio-Rad) thermal cycler.

c. Library Preparation

DNA was diluted to 1ng/uL with nuclease-free water in preparation for sequencing. Library preparation was performed at the sequencing laboratory of the Division of Human Genetics of UCT, using the Ion AmpliSeq Kit for Chef DL8 according to manufacturer's protocol (https://tools.thermofisher.com/content/sfs/manuals/MAN0013432Ion_AmpliSeq_Library_Prep_on_Ion_Chef_UG.pdf). From this step of the procedure, only sixteen (n=16) DNA samples were includedas per the Ion AmpliSeq Kit for Chef DL8 protocol. The selection of samples to be finally included was based on their quality, purity and integrity. The remaining DNA samples were archived for future sequencing. The steps followed are shown in Figure 5.

Preparation of the genomic DNA samples (n=16)

V

Creation of a sample set on the Ion Torrent Server

V

Addition of amplicons pools to positions A and B of the reagents cartridge

V

Addition of15ng genomic DNA to the Ion Code barcode plate

V

loadingand initialisation ofthe Ion Chef instrument

V

Ion Chef run initiation

V

Unloading of the Ion Chef Instrument at the end of the run, after 7 hours

Figure 4: **Workflow summarizing library preparation on the Ion Chef**

d. Template Preparation

The Ion Chef was used for templating of the Ion Sphere particles, clonal amplification and loading of the sixteen libraries on a 316 PGM chip (Ion PGM Hi-Q Chef Kit).

e. DNA Sequencing

Sequencing was performed on the Ion PGM (Thermo Fisher Scientific) (see run summary in Figure 5).

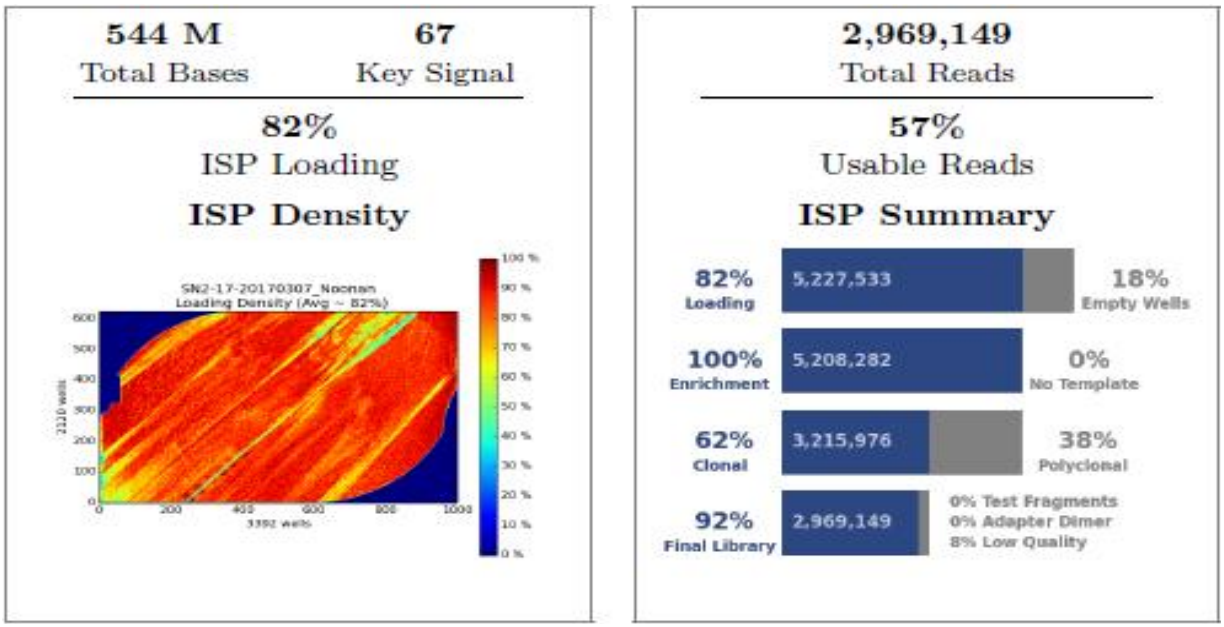

Figure 5: **Run summary**

Note,82% loading efficiency of the PGM 316 chip, of which 62% of the ion sphere particles were clonal. After the removal of 8% low quality reads, a final number of almost 3 GB of usable reads was obtained.

2.3.4 BIOINFORMATICS ANALYSIS

The Ion Reporter bioinformatic pipeline of the Ion Torrent Suite and the Ion Reporter cloud-based software (ThermoFisher Scientific) was used to perform basic workflow for NGS, including quality assessment, readalignment, variants identification, variant annotation, visualisation, prioritisation, and filtering.From the usable reads, 99% could be mapped to the human reference genome used (Homo sapiens, hg19, build 37.2). Owing to the challenges in NGS data analysis and interpretation, additional bioinformatic analysis was executed including variant filtering, prioritisation and interpretation, based on the VCF file generated by the Ion Reporter software. This was achieved with bioinformatics support from a research officer at the genomic laboratory of the Division of Human Genetics of UCT.Manual filtering and prioritisation of generated variants was performed using:

- Filtered variant frequencies for our dataset: only variants with a frequency < 0.75 in our dataset were selected, assuming that variants

present in the dataset with a frequency > 0.75 would be likely sequencing errorsthan real disease-causing variants;

- Zygosity of variants: homozygous variants were systematically excluded as NS is caused by heterozygous variants;
- Variant's function: synonymous variants were systematically excluded as no change in protein function was anticipated;
- Variants labelled "non-pathogenic" according to ClinVar by the Ion Reporter software were excluded;
- Variants with a minor allele frequency (MAF)≥0.01 based on the 1000 genome project and 5000 exomes were excluded as they are likely polymorphism than true disease-causing variants;
- Variant's location: only exonic variants were selected. Intronic as well as and 5', 3' UTR variants were excluded.

The pathogenicity of each selected variant was subsequently assessed using online genomic softwares (www.clinvar.com;www.decipher.sanger.ac.uk; IGV viewer; www.mutationtaster.org; www.ensembl.org), existing online databases for NS (www.nseuronet.com) and published literature on NS-associated variants for each selected gene of our panel. Intronic variants and variants located in intron/exon boundaries were not specially investigated.

2.3.5 SANGER SEQUENCING VALIDATION

Due to the financial and time limitations, pathogenic variants identified by NGS could not be validated with Sanger sequencing and/or in silico pathogenicity tools analysis in this study. This will be done in the future.

2.4 GENOTYPE-PHENOTYPE CORRELATIONS METHODS

The relationship between the genotype and the phenotype of all mutation-positive patients was established as follows:

- The phenotype of each mutation-positive patient was reviewed, including photographs when available;

- Phenotypes were grouped by genes involved and comparative description of NS key clinical characteristics was achieved, based on international medical literature.

2.5 STATISTICAL ANALYSIS

A questionnaire constructed in English, with Afrikaans and Xhosa translations was administered to each selected proband. All statistical analysis was performed using STATA (Version 13.1; Stata Corp, College Station, Texas, USA).

- Descriptive statistics

Descriptive statistical analysis was used to characterise the total sample and relevant genotype-phenotype correlations. Continuous variables were described using means (standard deviations) or medians (interquartile ranges) depending on their distributions, while categorical variables were described as frequencies and percentages.

CHAPTER 3

RESULTS

3.1 SOCIODEMOGRAPHIC DATA

From January 2015 to January 2017, a total of twenty six (n=26) patients were included in this study. The majority werechildren (n=20; 77%) with a median age at clinical diagnosis of 4.5 years (range: 1month - 51years) (Figure 6). There was a slight predominance of males with 57.7% (n=15) of males against 42.3% (n=11) of females (sex ratio: 1.36).

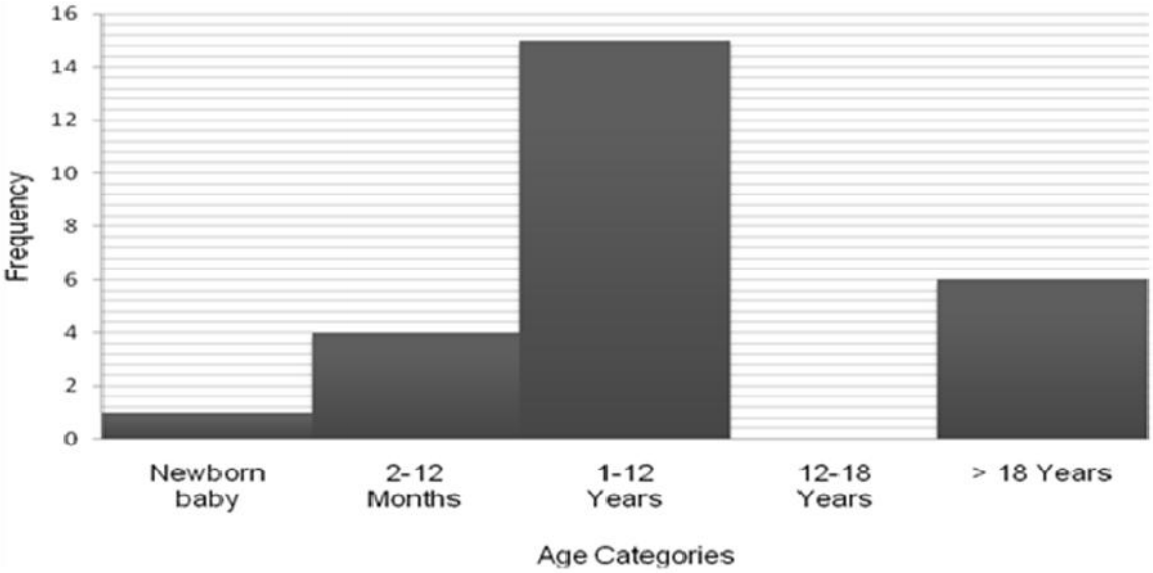

Figure 6: Age distributionin the cohort of 26 patients with a clinical diagnosis of NS

The majority of patients was clinically diagnosed in childhood (1-12 years) (n= 15; 57.7%). Only one patient was diagnosed in neonatal period. There was a gap between childhood and adulthood with no patient diagnosed in adolescence.

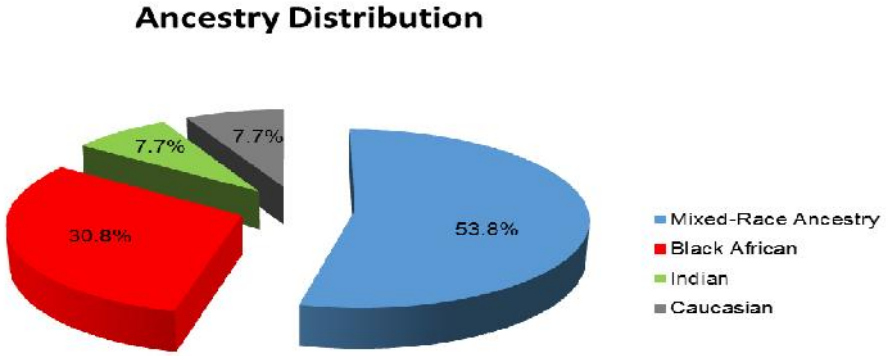

Figure 7: Ancestry distribution in the cohort of 26 patientswith a clinical diagnosis of NS.

Individuals of mixed-ancestry background were the most represented group (53.8%), followed by black Africans (30.8%).

3.2 CLINICAL DATA

3.2.1 FAMILY HISTORY

Half (n=13) of patients had a family history suggestive of NS, while 42.3% (n=11) of patients were likely simplex cases. The family history was unknown in two adopted patients. Of the 13 patients with a family history of NS, 9 were unrelated.

3.2.2 PHENOTYPIC DESCRIPTION

3.2.2.1 Antenatal Features

Antenatal abnormalities were present in 23.1% (n=6) of patients (Table 6), while no abnormalities were found in 50% (n=13) of patients. Antenatal data was not available in the remaining cases (n=6).

Table6: **Antenatal abnormalities**

Type of abnormality		Frequency (n=26)	Proportion (%)
Polyhydramnios		2	7.7
Increased Nuchal Translucency		1	3.8
Intrauterine Growth Restriction (IUGR)		1	3.8
Short Limbs		1	3.8
Renal abnormalities	Multicystic kidney	1	7.7
	Pelvi-ureteric Junction (PUJ) obstruction	1	

3.2.2.2 Birth Parameters

Prematurity was recorded in 15.4% (n=4) of cases.The mean birth weight was 2582.65g ±1276.1SD (range: 2080-4420), with the majority of patients (90.9%) having a birth weight above 2500g. The birth length of patients was not available in up to12 cases (46.1%); in the remainingcases (n=14), the mean birth length was 48.82 ±3.1SD (range: 43-54).

3.2.2.3 Developmental Milestones

Gross motor milestones were on par for the majority of patients, with ability to walk before the age of 18 months reported in 61.5% (n=16) of cases. Six (23.1%)patients

were unable to walk after 24 months. Speech delay was reported in 50% (n=13) of cases, and information was not available for 6 patients (23.1%).

3.2.2.4 Medical History

One or more major structural defects involving an organ system were present in 69.2% (n=18) of cases. Structural defects involving organ systems other than the cardiovascular system are listed in Table 7.

Table7: **List of the major structural defects in 26 Patients with NS**

Structural defect	Frequency (n=26)	Proportion (%)
Right pulmonary hypoplasia with elevated right diaphragm	1	3.8
Agenesis of the corpus callosum	1	3.8
Fused ribs	1	3.8
Multicystic kidney	1	3.8
Duplex Left collecting system	1	3.8
Undescended testes	7	46.7 males
Pyloric stenosis	1	3.8
Umbilical hernia	1	3.8

3.2.2.5 Surgical History

A total of 19 (73.1%) patients had undergone at least one surgical procedure since birth, cardiovascular surgery being the most common (42.3%), followed by orchidopexy (11.5%). Indications for surgery are summarized in Table8.

Table 8:**Indications for Surgery**

Surgery	Frequency (n=26)	Proportion (%)
Heart defect	11	42.3
Undescended testes	3	11.5
Bilateral carpal tunnel syndrome	1	3.8
Tonsillectomy	1	3.8
Desmoid cyst	1	3.8
Spontaneous pneumothorax	1	3.8
Inguinal hernia	1	3.8
Umbilical hernia	1	3.8
Pyloric stenosis	1	3.8
Appendicitis	1	3.8
PEG tube insertion (swallowing difficulties)	1	3.8
Eye surgery (strabismus)	1	3.8

3.2.2.6 Craniofacial Features

The craniofacial features associated with NS were widely variable among our patients. Some patients presented withtypical craniofacial dysmorphism (Figure 6), while others had a rather mild craniofacial phenotype. The most frequent feature was epicanthic folds, present in65.4% of patients, followed by low-set ears (57.7%). The craniofacial features found in our cohort are summarized in Table 9. Comparisons of the craniofacial features by age and ethnic groups are shown in Tables 10 & 11.

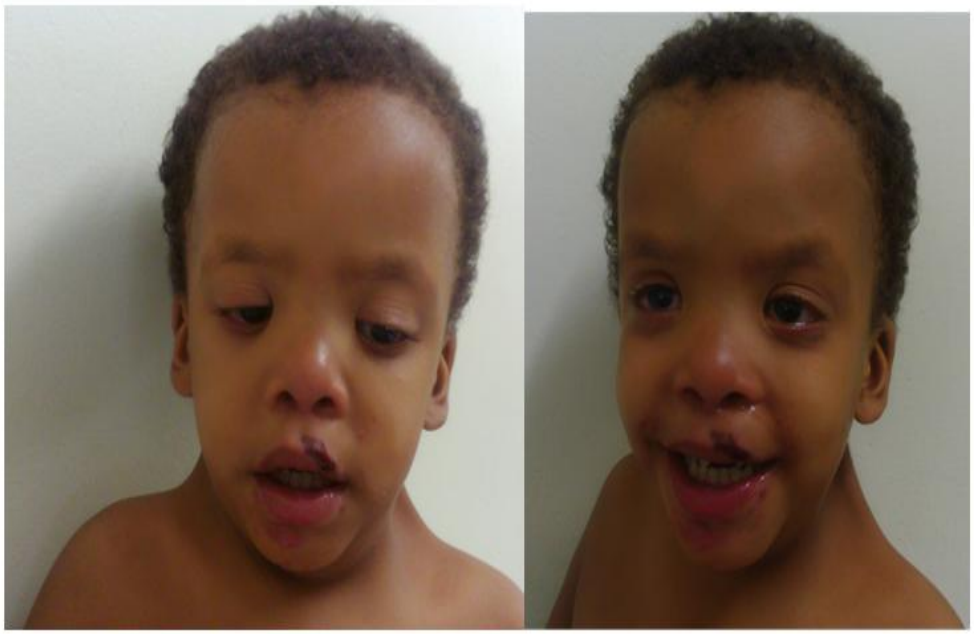

Figure8:**Face and Neck of a 3-year-old boy with NS.**
Typical craniofacial dysmorphic features occurring in children with NS.
Note: Elongated face;impression of large head;curly hair; widely spaced eyes; epicanthic folds; ptosis;tall forehead;low-set ears; short, wide and depressed nasal root; short and webbed neck.

Table 9: **Summary of Craniofacial Features**

Features		Frequency (n=26)	Proportion (%)
Forehead & face	Tall & prominent forehead	12	46.1
	Elongated face	12	46.1
	Coarse face	5	19.2
	Myopathic face	5	19.2
	Macrocephaly	4	15.4
	Thin & transparent skin	2	7.7
	Epicanthic folds	17	65.4
	Ptosis	12	46.1
	Widely spaced eyes	8	30.8
	Downslanting palpebral fissures	7	26.9

Eyes	Thick hooded eyelids		6	23.1
	Blue green irises		3	11.5
	Arched and diamond-shaped eyebrows		3	11.5
	Others	Upslanting palpebral fissures	1	3.8
		Deep-set eyes	1	3.8
Ears	Low set		15	57.7
	Posteriorly rotated		14	53.8
	Thick helices		12	46.1
	Prominent lobe		1	3.8
Nose	Short and broad		13	50
	Depressed root		13	50
	Upturned tip		7	26.9
	High & thin bridge		5	19.2
	Prominent noso-labial fold		3	11.5
Mouth	High wide picks of the vermilion		10	38.5
	Micrognathia		8	30.8
	Deeply grooved philtrum		6	23.1
	Long philtrum		1	3.8
Neck	Short neck		12	46.1
	Webbing		5	19.2
	Excessive nuchal skin		2	7.7
Other	Malar hypoplasia		1	3.8

Table 10: Comparison of craniofacial features between age groups

Characteristic craniofacial features were less distinctive in new-borns (n=1) and adults (n=6). Features were more characteristic in infants (2-12 months), with the most common being widely spaced eyes, epicanthic folds, ptosis and broad nose with depressed nasal root.

Features	New-born **(n=1)**	2-12 months **(n=4)**	1-12 years **(n=15)**	>18 years **(n=6)**
Macrocephaly	1(100%)	2(50%)	0	0
Tall and prominent forehead	1(100%)	2(50%)	8(53.3%)	1(16.7%)
Coarse face	0	1(25%)	2(13.3%)	5(83.3%)
Elongated face	1(100%)	1(25%)	6(40%)	4(66.7%)
Widely spaced eyes	0	3(75%)	5(33.3)	0
Epicanthic folds	1(100%)	3(75%)	9(60%)	3(50%)
Ptosis	0	3(75%)	10(66.7)	0
Low-set ears	1(100%)	2(50%)	9(60%)	3(50%)
Short, broad, depressed nasal root	0	3(75%)	12(80%)	3(50%)
Prominent naso-labial folds	0	0	2(13.3%)	2(33.3%)
High wide peaks of the vermilion	0	2(50%)	11(73.3)	2(33.3%)
Short neck	0	2(50%)	8(53.3%)	2(33.3%)
Webbed neck	0	1(25%)	3(20%)	1(16.7%)

Table 11: Comparison of key dysmorphic features between ethnic groups

Key dysmorphic featureswere less marked in Caucasian patients (n=2) followed by Indians (n=2). Black Africans presented with the most dysmorphic features. Epicanthic folds, ptosis and low-set ears were most common in Black Africans compared to other ethnic groups. Short stature was present in >87% of patients in all ethnic groups, except for Caucasians who all had a normal height.

Features	Black African **(n=8)**	Coloured **(n=14)**	Caucasian **(n=2)**	Indian **(n=2)**
Widely spaced eyes	2(25%)	5(35.7%)	0	1(50%)
Ptosis	6(75%)	7(50%)	0	0
Epicanthic folds	7(87.5%)	9(64.2%)	0	1(50%)
Low-set ears	6(75%)	7(50%)	1(50%)	1(50%)
Webbed neck	3(37.5%)	1(7%)	1(50%)	0
Short stature	7(87.5%)	12(87.5%)	0	2(100%)

3.2.2.7 Cardiovascular features

At least one cardiac abnormality was identified in 65.4% (n=17) of patients (Figure 7). The most common congenital heart defect was Pulmonary Valve Stenosis, found in 34.6% of cases.Hypertrophic Cardiomyopathy (HCM) was identified in 19.2% of patients and left axis deviation on ECG was found in 23.1% of cases.

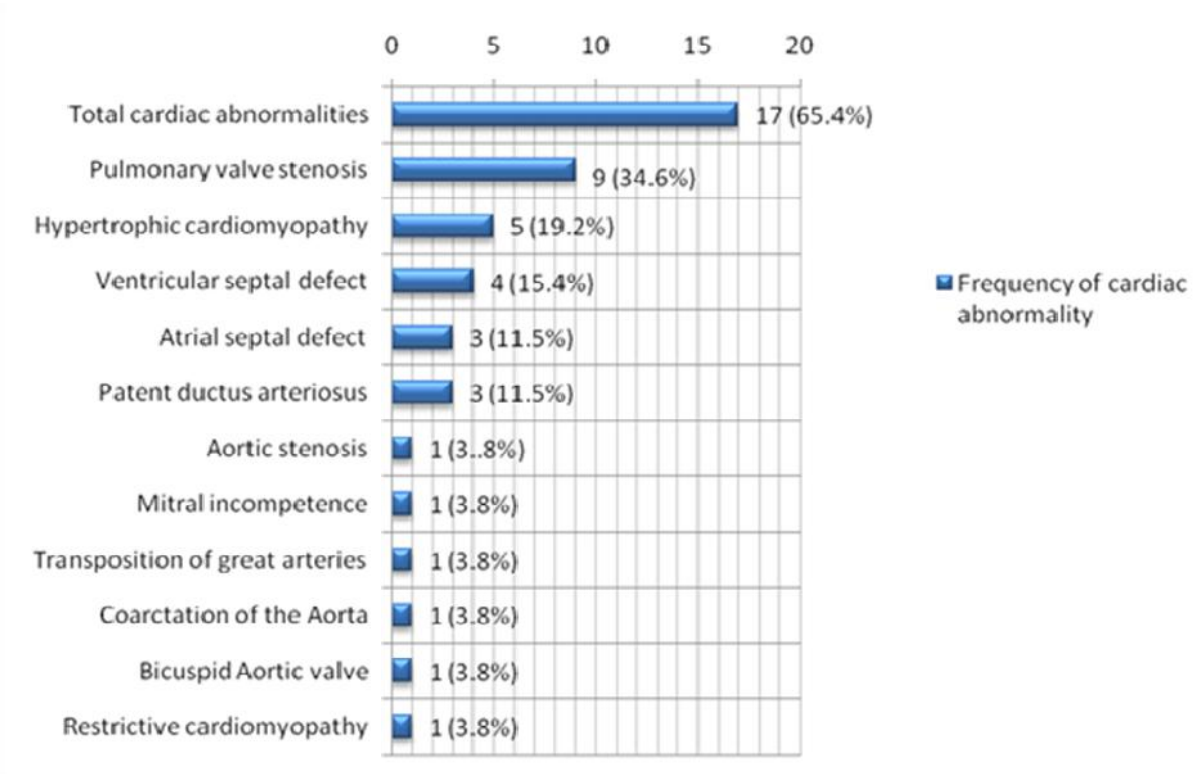

Figure 9: Profile of Cardiac Defects in 26 Patientswith Noonan Syndrome

3.2.2.8 Other clinical features

The clinical featuresfound in our cohort of NS patients,excluding cardiac abnormalities (Figure 7),are summarized in Table 12.

Table 12: **Organ-systems involvement**

Features identified			Frequency (n=26)	Proportion (%)
Short stature			21	80.8
Central Nervous System	Agenesis of the Corpus Callosum		1	3.8
Eye	Strabismus		4	15.4
	Myopia		3	11.5
	Nystagmus		2	7.7
	Astigmatism		1	3.8
Auditory	Conductive hearing loss		3	11.5
	Sensorineural hearing loss		1	3.8
Oral & Dental	High arched palate		11	42.3
	Dental malocclusion		11	42.3
	Dental caries		2	7.7
	Widely spaced teeth		1	3.8
	Overcrowded teeth		1	3.8
Chest	pectus excavatum (PE)		6	23.1
	Pectus carinatum (PC)		4	15.4
	PC superiorly & PE inferiorly		1	15.4
Musculoskeletal (other than chest)	Spine	Scoliosis	5	19.2
		Lumbar hyperlordosis	1	3.8
	Limbs	Cubitus valgus	2	7.7
		Talipes equinovarus	1	3.8
		High arched feet sole	1	3.8
	Joints	Hyperlaxity	3	11.5
		Contracture	2	7.7
	Other	5th finger clinodactyly	1	3.8
		Overlapping 2-3 toes	1	3.8
		Fused ribs	1	3.8
Gastrointestinal	Feeding difficulties	Poor suck & prolonged feeding time	3	11.5
		Severe feeding issues with tube-feeding for > 3 weeks	3	11.5
		Very poor suck & slow feeding with recurrent vomiting	2	7.7
	Pyloric stenosis		1	3.8
Genitourinary	Undescended testis		7/15 males	46.7
	Duplex left collecting system		1	3.8
	Multicystic kidney		1	3.8
	Thick curly hair		15	57.7
		Cafe-au-lait spots	9	34.6
		Pigmented naevi	3	11.5

Dermatology	Skin	Keratosis pilaris atrophicans faciei	1	3.8
		Axillary freckling	1	3.8
		Hypopigmented facial lesion	1	3.8
Haematology & Oncology	Bleeding diathesis (easy bruising)		14	53.8
	Abnormal PT & PTT		1	3.8
	Mild thrombocytopenia		1	3.8
	Solid tumour (Desmoid cyst)		1	3.8
Lymphatic	Lymphedema		3	11.5
Neurology, Cognition& Behaviour	Learning difficulties		11	42.3
	Mild intellectual disability		7	26.9
	Severe intellectual disability		1	3.8
	Behavioural issues	Hyperactive	5	19.2
		ADHD	4	15.4
		Self-injury	1	3.8

3.3 MOLECULAR GENETICDATA

Molecular genetic analysis wasexecuted on 16 genomic DNA samples out of the 26 collected. Targeted NGS of a panel of fourteen genes was performed in all selected samples using Ion Torrent instruments, with screening of all the exons, introns and intron-exon boundaries.

3.3.1 VARIANTS PROFILE

Of the 16 samples from apparently unrelated patients analysed, variants predicted to be pathogenic were detected in 7 (43.7%) cases (Table 13). These patients included 6 males (86%) and 1 female (14%). Five (5/7; 71%) were detected in patients with family history suggestive of NS and 2(2/7; 28%) were apparently *de novo*(Figure 8).

Table 13: Characteristics of the predicted pathogenic variants detected

Of the seven mutation-positive patients identified, 3(42.8%) had a pathogenic variant in *CBL*, 2(28.6%) in *PTPN11* and 2(28.6%) in *MAP2K1.*

Locus	Transcript	Gene	Exon	Nucleotide substitution	Amino acid substitution	Variant function	Case number
chr15:66729181	NM_002755.3	*MAP2K1*	3	c.389A>G	p.Tyr130Cys	missense	Case#10
chr15:66727484	NM_002755.3	*MAP2K1*	2	c.200A>C	p.Asp67Ala	missense	Case#15
chr11:119170290	NM_005188.3	*CBL*	16	c.2520T>G	p.Cys840Trp	missense	Case#7
chr11:119156193	NM_005188.3	*CBL*	11	c.1858C>T	p.Leu620Phe	missense	Case#13
chr11:119169161	NM_005188.3	*CBL*	15	c.2345C>T	p.Pro782Leu	missense	Case#14
chr12:112926890	NM_002834.3	*PTPN11*	13	c.1510A>G	p.Met504Val	missense	Case#2
chr12:112926876	NM_002834.3	*PTPN11*	13	c.1496C>T	p.Ser499Phe	missense	Case#3

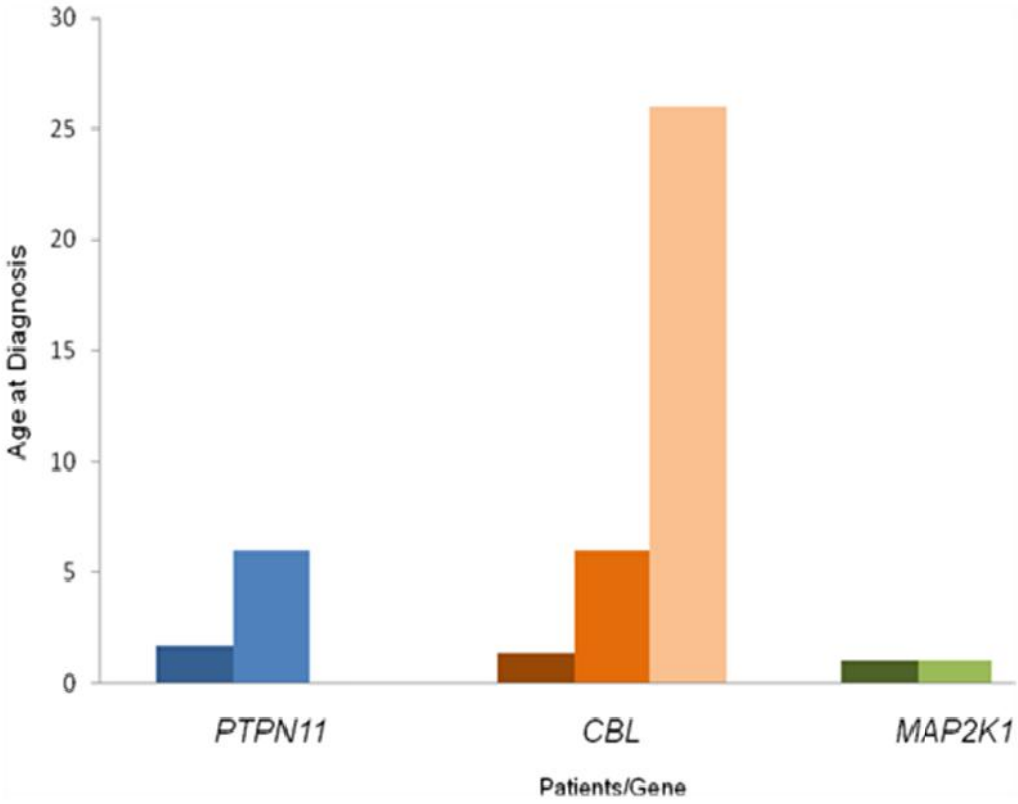

Figure 10: Age distribution in mutation-positive patients
The clinical diagnosis of NS was made in patients with pathogenic variants in *MAP2K1* earlier than in patients with pathogenic variants in *PTP11* and *CBL*.

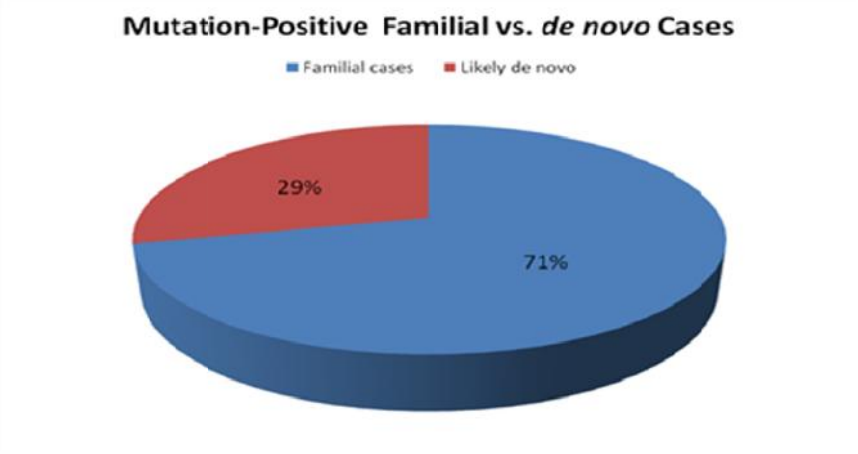

Figure 11: Family segregation of NS in patients (n=7) with positive results
The majority of patients (n=5; 71%) with a positive result after molecular analysis has a family history suggestive of a familial segregation of NS.

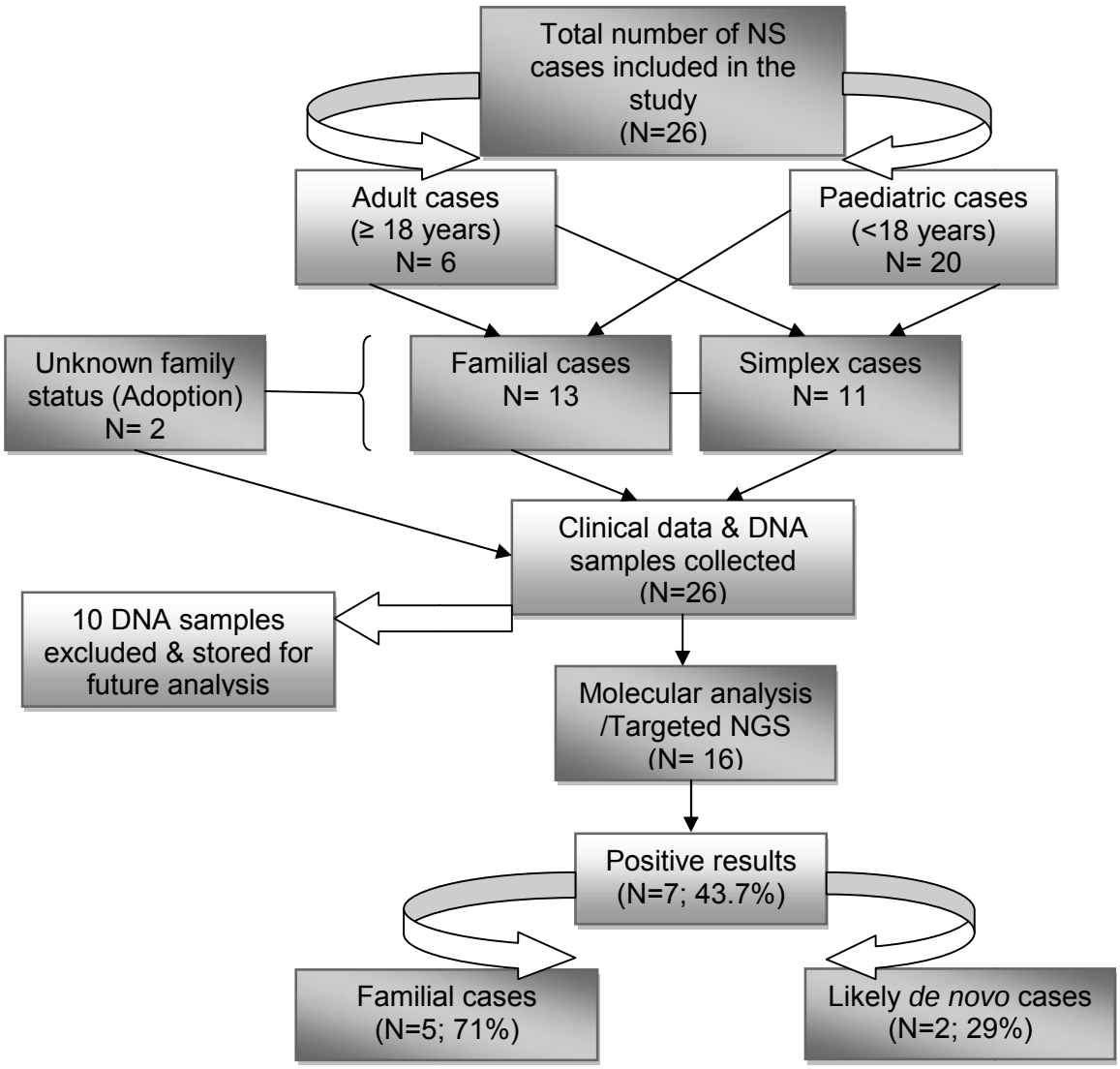

Figure 12: Summary of the clinical and molecular genetic data
This flowchart showssomesteps in our data processing.

3.4 GENOTYPE-PHENOTYPE CORRELATIONS

3.4.1 PHENOTYPE OF MUTATION-POSITIVE PATIENTS

The table below (Table 14) describes the association between pathogenic variants detected in our cohort and their related phenotype.

Table 14: Clinical characteristics of 7 patients with positive molecular testing

- *= Noonan syndrome; **= Cardiofaciocutaneous syndrome; ***= Intellectual disability; x= No abnormality; ?= Unknown; √= Abnormality present; N/A= Not applicable.
- The squares indicate features reported in the literature to be positively (black) or negatively (blue) associated with the presence of a pathogenic variant in each corresponding gene.

- The variant identified in Case#7 has been associated with Cardiofaciocutaneous Syndrome, a RASopathy sharing several features with NS.

	Case#1	Case#2	Case#3	Case#4	Case#5	Case#6	Case#7
Gene & Condition / **Phenotype**	***PTPN11*** NS*	***PTPN11*** NS	***CBL*** NS	***CBL*** NS	***CBL*** NS	***MAP2K1*** NS	***MAP2K1*** CFC**
Gender	M	M	M	F	M	M	M
Age at diagnosis	6y	20m	6y	26y	17m	12m	12m
Family history	√	√	√	√	x	√	x
Antenatal & birth features							
Polyhydramnios	x	?	x	?	x	x	√
Prenatal/neonatal lymphatic abnormalities	x	x	x	?	x	√	x
Birth weight (centile)	50th	10th	50th	?	<3rd	90th	25th
Birth length (centile)	50th	?	90th	?	<3rd	90th	?
Short stature (at diagnosis)	√	√	√	√	√	√	√
Craniofacial dysmorphism							
Typical facial features	√	√	√	x	x	√	√
Mild facial dysmorphism	x	x	x	√	√	x	x
Neck & Thoracic features							
Webbed /short neck	√	√	√	√	√	√	√
Pectus deformity of the chest	x	x	√	√	x	√	√
Congenital Heart Defect& ECG							
Pulmonary valve stenosis	√	x	x	x	x	√	x
Hypertrophic cardiomyopathy	x	√	x	√	x	x	x
Coarctation of the aorta	x	x	x	x	√	x	x
Left axis deviation on ECG	√	x	x	x	x	√	x
Aortic valve stenosis	x	√	x	x	x	x	x
Mitral valve incompetence	x	√	x	x	x	x	x
Bicuspid aortic valve	x	x	x	x	√	x	x
Neurology, Education& Behavioural Abnormalities							
Motor delay	x	√	x	?	x	√	√
Speech delay	x	x	√	?	N/A	√	√
Mild ID***	x	x	√	x	N/A	√	√
Learning difficulties	x	x	√	x	N/A	√	√
Hyperactivity	√	x	x	x	N/A	x	x
ADHD	x	x	√	x	N/A	x	x
Self-injury	x	x	x	x	x	x	√

Eye abnormalities							
Strabismus	x	x	x	x	x	√	x
Auditory abnormalities							
Conductive hearing loss	x	x	√	x	x	x	x
Renal abnormalities							
Undescended testis in males	√	x	√	N/A	x	√	x
Duplex collection system	x	x	x	x	x	√	x
Gastro-intestinal abnormalities							
Mild feeding difficulties	√	x	x	?	x	x	x
Severe feeding difficulties	x	x	√	?	x	x	√
Pyloric stenosis	x	x	√	x	x	x	x
Dermatologicabnormalities							
Cafe-au-lait spots	x	x	x	√	x	√	x
Pigmented naevi	x	x	x	x	x	x	√
Curly hair	√	x	√	x	√	x	√
Haematology& Oncology							
Bleeding diathesis	√	√	x	√	x	x	x
Abnormal PT& PTT	x	x	x	x	x	√	x
Desmoid cyst	x	x	x	x	x	√	x

3.4.2 COMPARATIVE DESCRIPTION OF CHARACTERISTICS

Table 15 presents and compares the mean age at diagnosis,as well as key NS clinical characteristics among mutation-positive patients with respect to the gene involved.

Table 15: Genotype-phenotype comparisons of the 3 genes identified

Patients with pathogenic variants in *MAP2K1* presented with more typical clinical features of NS, followed by patients with pathogenic variants in *PTPN11*. Clinical features were more discreet in individuals with pathogenic variants in *CBL*.

Characteristics	***PTPN11*** **(n=2)**	***CBL*** **(n=3)**	***MAP2K1*** **(n=2)**
Mean age at Diagnosis (year)	3.3	11.1	1
Positive family history	2(100)	2(66.7)	1(50)
Antenatal features	0	0	2(100)
Short stature	2(100)	3(100)	2(100)
Typical dysmorphic features	2(100)	1(33.3)	2(100)
Webbed/Short neck	2(100)	3(100)	2(100)
Pectus deformity of the chest	0	2(66.7)	2(100)
Congenital Heart Defects	2(100)	1(33.3)	2(100)
Pulmonary valve stenosis	1(50)	0	1(50)
Hypertrophic cardiomyopathy	1(50)	1(33.3)	0
Coagulopathy	2(100)	1(33.3)	1(50)
Skin features	0	1(33.3)	2(100)
Intellectual disability	0	1(33.3)	2(100)

CHAPTER 4

DISCUSSION, CONCLUSION AND FUTURE PERSPECTIVES

4.1 DISCUSSION

4.1.1 GENERAL COMMENTS

This study is, to the best of our knowledge, the first of its kind to investigate a cohort of NS individuals from clinical and molecular perspectives, with the use of NGS technology in South Africa. It provides insights into the spectrum of clinical features and pathogenic variants in South African individuals affected by NS.Furthermore, this study supports the necessity of establishing a local moleculardiagnostic testing for RASopathies in the public health sector in South Africa.

4.1.2 SOCIODEMOGRAPHIC CHARACTERISTICS

Sociodemographic analysis of the cohort revealed a male predominance. Of the 26 patients included in this study, 57.7% were males and 42.3% were females, with a sex ratio of 1.36. This is consistent with the results of a European family study on NS, which investigated 49 unrelated European families and showed a significant sex ratio bias in NS transmission (>1) favouring transmission to males(Tartaglia et al. 2004).Similarly, in a study of cardiovascular features in 293 individuals affected with NS, Prendiville and collaborators reported that 60% of the affected patients were male(Prendiville et al. 2014).

Individuals of mixed race ancestry were the most represented ethnic group (53.8%), followed by black Africans (30.8%), in keeping with ancestrydistribution in the Western Cape Province where the population is made up of 48.7% of individuals of mixed-race ancestry and 32.8% of black Africans (Statistics South Africa,Census 2011).

In our cohort, the age atclinical diagnosis of NS ranged from 1 month to 51 years with a median age at diagnosis of 4.5 years. The most represented age group was 1-12 years. Only one individual was diagnosed in the neonatal period and no diagnosis was made in the age group 12-18 years.This result may be explained by at least three factors: Firstly, in the local environment, not all pregnant womenhave ultrasound scans throughout pregnancy. For those in the cohort who have had one or more ultrasound scans, either no abnormality was found (50%), or even when prenatal features suggestive of NS were present(e.g. polyhydramnios, increased nuchal translucency, renal anomalies or Pulmonary Valve Stenosis),possible

association with NS was not always established by clinicians.In the absence of prenatal molecular diagnostic testing for NS in South Africa, recognition of prenatal features of NS could at least have contributed to increasethe proportion of patients diagnosed in the neonatal period. In fact, with a prenatal detection rate of 17.3%, Croonen and collaborators recommended NS testing in the presence of one or more of lymphatic abnormalities, renal anomalies, polyhydramnios and cardiac anomalies, in addition to an increased nuchal translucency(Croonen et al. 2013). Secondly, inthelocal setting, the limited clinical diagnostic ability of many clinicians untrained in dysmorphology, coupled with the scarcity of trained medical geneticists often results in initial misdiagnosis, followed bya late diagnosis, typically in childhood (1-12 years), after several hospital admissions and referral to medical genetics. Finally, in view of the age-relatedvariability in NS physical features, with subtlety of craniofacial features in many adults (Van der Burgt 2007; Roberts et al. 2013), moderate-to-severely affected individuals would be expected to be diagnosed by childhood, and mildly affected individuals in adulthood following either cardiac decompensation or cascade screening after the birth of an affected child. In our cohort, all adult patients were diagnosed with NS during their assessment in the Cardiology department.

4.1.3 FAMILY SEGREGATION

It is widely known that an average of 60% of individuals affected with NS have, in prospect or retrospect, a positive family history of NS. In our cohort, 50% of patients had a family history suggestive of NS, in agreement with the literature(Allanson & Roberts 2001).

4.1.4 PHENOTYPE

DEVELOPMENTAL PHENOTYPE

Developmental milestones were variable among our patients. Achievement of gross motor milestones was globally earlier than language milestones.In this study, 61.5% of patients were able to walk by the age of 18 months, slightly above the average of 21 months reported in a large study of the natural history of NS conducted by Sharland and collaborators(Sharland et al. 1992). Half (50%) of our patients were able to speak simple two-word sentences before the age of 24 months. A recent systematic review of the neuropsychological functioning of individuals affected with

NS, reported an average age of simple two-word sentences from 31 to 32 months (Pierpont 2016). The difference between our results and the literature may be explained by both the small size of our cohort and the fact that only screening measures were used. Application of comprehensive diagnostic assessments may have produceddifferent results.

FACIAL DYSMORPHOLOGY

Recognition of dysmorphic features can be very powerful in the clinical diagnostic process of NS (Van der Burgt 2007). The dysmorphology in NS varies with age and somewhat between ethnic groups. In a recent international collaborative study investigating NS-associated dysmorphic features in 125 individuals from diverse populations, we have demonstrated that regardless of the age and ethnic background, clinical findings in NS are similaracross ethnic groups. The three most common dysmorphic features, present in >70% of individuals were: widely spaced eyes (≥80%), low-set ears (>80%) and short stature (>70%). The two features found different among ethnic groups were ptosis and webbed neck. Ptosis was less common in black Africans (63%) while webbed neck was less common in Asians (Kruszka et al. 2017). The present study included black Africans, Caucasians, Indians and mixed-race ancestry individuals. The most common feature was epicanthic folds (65.4%), followed by low-set ears (57.7%). Widely spaced eyes were present in only 30.8% of patients, ptosis in 46.1% and short stature in 80.8%. Black Africans were found to have the most distinctive features; epicanthic folds, ptosis and low-set ears were most common in black Africans compared to other ethnic groups.Our small cohort size could explain the statistical difference between this study and the previously reported study (Kruszka et al. 2017). Comparison of features between age groups showed that typical craniofacial features were less distinctive in the neonatal periodand adulthood and more characteristic in infancy (2-12 months), in accordance with the medical literature (Roberts et al. 2013). A special point to note on the dysmorphology findings in this study is the high frequency of epicanthic folds, which maysimply represent a confounding factor. In fact, previous studies have reported epicanthic foldsto be very common in the general black South African population (Christianson et al. 1995).

CARDIOVASCULAR PHENOTYPE

NS is the second most common cause of syndromic congenital heart defects (CHDs) after Down Syndrome (Marino et al. 1999). CHDs are reported in 50-80% of individuals affected with NS (Hickey et al. 2011; Prendiville et al. 2014). Early detection and management of cardiovascularanomalies arecritical as they representthe major cause of mortality. In our cohort at least one CHD was identified in 65.4% of patients. Pulmonary Valve Stenosis was the most common CHD as reported in previous studies, but with a lower incidence (34.6%).In a retrospective and descriptive study conducted by Prendiville and collaborators in Boston, USA, investigating the spectrum of cardiac anomalies in 293 NS patients, CHD was reported in 81% of patients. Pulmonary Valve Stenosis was the most common CHD, reported in 57% of patients and HCM was reported in 16% of patients.

The lower incidence of Pulmonary Valve Stenosis in our study could be well explained by the small size of our cohort. However, it could be a specificityof the South African population in relation to the genotype of affected individuals with NS in this environment.There is a thought that bias may exist in the published frequency of CHD in NS as, in the past, the presence of CHD was required by many clinicians to establish the clinical diagnosis of NS (Allanson & Roberts 2001).We believe that studying a larger South African cohort mayaid to make a more convincing conclusion.

4.1.5 MOLECULAR ANALYSIS

In order to maximize the detection rate of NS in the cohort investigated in this study, we used the approach of targeted NGS, on the Ion PGM platform with Ion AmpliSeq Noonan Research Panel (ThermoFisher Scientific, USA). This multigene panel allowed for simultaneous sequencing of fourteen causative genes known to be associated to NS, based on recent medical literature (Nelen et al. 2014). A total of sixteen DNA samples from 16 unrelated patients were analysed. Seven variants predicted as pathogenic were detected in 3 genes (7/16 = 43.7% of detection rate), including *CBL* (3/16 = 42.8%), *PTPN11* (2/16 = 28.6%) and *MAP2K1* (2/16 = 28.6%).All the variants detected were missense variants, in keeping with previous

studies (Tartaglia et al. 2002; Nava et al. 2007; Martinelli et al. 2010). The detection rate of >70% is anticipated when using a comprehensive multigene panel testing or WES (Aoki et al. 2016). In our cohort, however, the detection rate was 43.7% (7/16), which is similar to that obtained(47.5%) in a comparable study in Italy (Lepri et al. 2014). It is unlikely that inappropriate phenotyping is the reason for our detection rate (<70%), in view of the particularly stringent patient selection process applied. Therefore, some patients presenting with typical clinical features of NS had unexpectedly negative molecular test results. Furthermore, the mutation frequency in the genes screened in this study, with *CBL*found to be the most frequently mutated gene, is globally deviated from the results of previous similar series. In fact, since the first report of a molecular NS study by Tartaglia and collaborators in 2002, *PTPN11* has been consistently reported as the most commonly mutated gene in NS, explaining 50-60% of all NS cases in other population groups(Tartaglia et al. 2002; Lepri et al. 2014).*CBL*isreported in the literature as a rare cause of NS, accounting for < 3% of cases(Martinelli et al. 2010), and *MAP2K1* is reported to account for about 4.2% of NS cases(Nava et al. 2007). The global difference in the mutation frequency in our study compared to the literature may be explained by the small size of our cohort. However this result may highlight a specificity of the South African population.

Interestingly, one pathogenic *MAP2K1* variant (c.389A>G; p.Tyr130Cys) in our cohort(Table 13, case#10) has been frequently associated with CFC syndrome in the published literature (Rodriguez-Viciana et al. 2006).This patient was initially diagnosed with NS at 12 months of age but the diagnosis was subsequently changed to Costello syndrome (CS) with the progression of clinical features. This illustrates the challenges of clinical diagnosis of some NS patients, due to overlapping features with other RASopathies, especially CFC syndrome and CS.

Mutation-positive patients in our study includedunrelated familial caseswith a proportion of 71%, in agreement with the literature (Allanson & Roberts 2001).

4.1.6 GENOTYPE-PHENOTYPE CORRELATIONS

Genotype-Phenotype correlations have been established for someNS-associated genes, *PTPN11* being the most studied gene.Variability in phenotypic expression, high genetic heterogeneity and low mutation frequency in a number of NS genes are

among the difficultiesin establishing comprehensive correlations between known causative genes or variants and specific phenotypes. In the present study, by comparing the phenotype associated withthe genes or variants detected based on twelve key clinical features of NS (Table 15), we found that clinical features were more characteristic in patients with pathogenic variants in *MAP2K1*(8/12), followed by those with pathogenic variants in *PTPN11* (6/12). Patients with pathogenic variants in *CBL* had less distinctive clinical characteristics. Specifically, an antenatal feature was present in all the patients with pathogenic variants in *MAP2K1* compared to patients with pathogenic variants in *PTPN11* and *CBL* who had no antenatal features identified. The above findings could explain the early mean age at clinical diagnosis in patients with *MAP2K1* pathogenic variants (1year) compared to patients with pathogenic variants in *PTPN11* (3.3years) and *CBL* (11.1years) in our study. CHDs were present in only 33.3% (1/3) of patients with pathogenic variants in *CBL*, none of them presenting with pulmonary valve stenosis. No patient with a *MAP2K1* pathogenic variant was found to have HCM.

Genotype-phenotype studies in *PTPN11*have reported positive association between short stature, pulmonary valve stenosis, coagulopathy, pectus deformities of the chest, and *PTPN11*pathogenic variants. However HCM has been reported as negatively associated to *PTPN11*pathogenic variants(Tartaglia et al. 2002; Yoshida et al. 2004a). With regard to *PTPN11* in our study, withthe exception ofHCM which was present in 50% (1/2) of patients and pectus deformities of the chest, absent in the two patients, our result (Table 15) is in keeping with the literature.

All the patients with pathogenic variants in *MAP2K1* had typical facial dysmorphic features and skin manifestations of NS in agreement with previously published studies (Nava et al. 2007; Nyström et al. 2008).

To date, very little is known about genotype-phenotypeassociations in NS with pathogenic variants in *CBL*. In an Italian study, Martinelliand collaborators (Martinelli et al. 2010)reported on 4 individuals (2 sporadic and 2 familial cases) with NS and pathogenic variants in*CBL*. Although pectus deformities of the chest, stature >3rd centile and short or webbed neck were frequently present, the clinical features of the affected individuals were very variable. Interestingly, one of the patients described presented with mitral valve insufficiency, which was also found in one of our patients with pathogenic variants in *CBL*.

4.1.7 LIMITATIONS OF THE STUDY

We acknowledge that the present study is not without limitations: 1- With the small size of our cohort, the results may not necessarily be inferable to the study population. 2- This is a hospital-based study with a likely ascertainment bias. The NS phenotype is highly variable in general, even within the same family. Therefore, individuals with a mild phenotype (e.g. absence of CHD) may not necessarily seek medical attention and go undiagnosed. Of the six adult patients included in this study, only two did not present with a cardiac anomaly; the remaining four adult patients were enrolled during cardiologic assessment of late onset cardiovascular anomalies strongly associated with NS. 3- Molecular investigations could not be offered to all the patients included in the study, potentially reducing the power of our results. 4- Sanger sequencing validation of the detected pathogenic variants was not performed because of time limitations. Therefore, the presence of false positive results, while unlikely, could not be excluded. However, some pathogenic variants detected have been previously reported in the medical literature. All variants detected were subjected to careful analysis with the available *in silico* variant annotation and pathogenicity predictor tools.

4.2 CONCLUSIONAND FUTURE PERSPECTIVES

This study represents,to the best of our knowledge, the first clinical and molecular researchin South African individuals affected with NS.

Our results show that a number of South African paediatric and adult patients are affected with RASopathies, in particular NS. Clinical characteristics found in affected individuals are globally similar to those reported in other populations, and careful phenotyping based on internationally available diagnostic criteria can effectively facilitate the diagnosis of most NS-affected individuals in South Africa. This study also demonstrates that targeted NGS with multigene panel testing is a powerful diagnostic method, which can be successfully applied to the molecular diagnosis of NS and related conditions in South Africa. The use of targeted NGS allowed for detection of pathogenic variants in genes infrequently associated with NS in other populations, which could be missed with the traditional approach or with single gene screening by Sanger sequencing. While variant distribution in South African NS

patients may be different from that previously reported in other population groups, the results of the genotype-phenotype correlations attempted in this study appear to support the existing data. Study of a larger South African NS cohort is warranted in order to comfortably infer these results. Finally, our results support the clinical utility of molecular testing in NS, which can aid early diagnosis, cascade screening, appropriate genetic counselling and management.Identification of common pathogenic variants underlying NS and related conditions in South Africans has the potential to facilitate early diagnosis and allow the study of specific genotype-phenotype correlations, which in turn can aid clinical diagnosis and support the development of future therapeutics.

Further work to complete certain aspects of this study is required. This includes Sanger sequencing validation of all putative pathogenic variants found and a more in-depth bioinformatics analysis of the NGS files of individuals in whom no exonic variants were detected, with specific focus on the intron-exon boundaries.
In addition, cascade screening of affected families will be performed and clinical management arranged when needed. The results of the present study will be used as support for our plan to establish molecular diagnostic testing for the RASopathies in the public health sector in Cape Town.

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APPENDICES

Appendix 1: DATA SHEET

CODE……../FN……

PLACE: DATE:

I- SOCIO-DEMOGRAPHIC DATA

1- Age: DOB: Age at diagnosis: ……

2- Race: Black African = 1 Caucasian = 2 Coloured = 3 Others = 4

3- Sex: Male = 1 Female = 0

4- Country of origin: South Africa = 1 other = 0

5- Occupation: scholar/student = 1 = 2 unemployed = 3 employed part-time =4
Employed full-time = 5

6- Education: tertiary = 1 secondary = 2 Primary = 3 No education = 4

7- Marital status: single = 1 Married= 2 partnership = 3 divorced = 4
Widow/widowed = 5

8- Religion : Christian = 1 Muslim = 2 other = 3 Not religious = 4

II- FAMILY HISTORY / PEDIGREE

III- PERINATAL HISTORY

A. PRENATAL HISTORY

1- Maternal illness : No = 0 Yes = 1 unknown = 2

If yes specify ……. ……..... = 3

2- Teratogens: Alcohol = 1 illicit drugs = 2 medication = 3

If 2 or 3 specify ……... = 3 unknown = 4

3- Others: Yes (specify)…… = 1 No = 0

4- Prenatal ultrasound: normal = 1 abnormalities = 2 No US = 3

Unknown = 5 If 2 specify…….. = 4

B. BIRTH

1- Term of the pregnancy: < 37 wks = 1 ≥ 37 wks = 2 unknown = 3

2- Birth weight…… Length…….. Head circumference……..

3- Complications: No = 0 Yes = 1 unknown = 2

If 1 specify…….

IV- DEVELOPMENTAL HISTORY

1- Sat: < 1y = 1 > 1y = 2 unknown = 3 N/A = 4

2- Walked: < 15 m = 1 15-24m = 2 > 24 m = 3 unknown = 4 N/A = 5

3- Talked: < 24 m = 1 ≥ 24 m = 2 unknown = 3 N/A = 4

V- PAST MEDICAL HISTORY

A. MEDICAL

1- Structural defect(s): No = 0 Yes = 1 unknown = 2

If yes specify …….

2- Functional defect (s): No = 0 Yes = 1 unknown = 2

If yes specify …….

3- Metabolic defect (s): No = 0 Yes = 1 unknown = 2

If yes specify …….

4- Other: No = 0 Yes = 1

If yes specify …….

B. SURGICAL

1- History of surgery: No = 0 Yes = 1 unknown = 2

2- Type of surgery: brain = 1 eye = 2 chest = 3 heart = 4 abdominal = 5
Spine = 6 hernia = 7 limb (s) = 8 unknown = 9 other = 10 N/A = 11

VI- CLINICAL DATA

A. CRANIOFACIAL DYSMORPHISM

1- **Forehead, face**: Tall & prominent forehead = 1 low post hairline = 2
Macrocephaly = 3 elongated face = 4 coarse face = 5 myopathic face = 6
Thin & transparent skin = 7 others (specify)……. = 8

2- **Eyes**: hypertelorism = 1 downslanting palp fissures = 2 epicanthal folds = 3
Ptosis = 4 thick hooded eyelids = 5 blue green irises = 6 arched and diamond-shaped eyebrows = 7 others (specify)…… = 8

3- **Ears**: Low-set = 1 post rotated = 2 thick helices = 3 others…….. = 4

4- **Nose**: short & broad = 1 depressed root = 2 upturned tip = 3
High & thin bridge = 4 prominent nasolabial fold = 5 others ……. = 6

5- **Mouth**: deeply grooved philtrum = 1 high wide peaks of the vermilion = 2
Micrognathia = 3 others (specify)…….. = 4

6- **Neck**: Excessive nuchal skin = 1 Webbing = 2 others……. = 3

7- Others: No = 0 Yes (specify)…… = 1

B. GROWTH

1- Short stature**:** Yes = 1 No = 2

C. CENTRAL NERVOUS SYSTEM

1- Arnold-Chiari malformation type I: No = 0 Yes = 1 Unknown = 2

2- Hydrocephalus: No = 0 Yes = 1

3- Others: No = 0 Yes (specify)…… = 1

D. OPHTHALMOLOGY

1- **Strabismus:** No = 0 Yes = 1

2- **Anterior segment changes:** No = 0 Yes = 1

3- **Refractive errors**: No = 0 Yes = 1

4- **Fundal abnormalities:** No = 0 Yes = 1

5- **Amblyopia**: No = 0 Yes = 1

6- **Nystagmus:** No = 0 Yes = 1

7- **Others:** No = 0 Yes = 1(specify)……

E. AUDITORY

1- Sensorineural hearing loss: No = 0 Yes = 1
2- Hearing loss secondary to recurrent otitis media: No = 0 Yes = 1
3- Conductive hearing loss: No = 0 Yes = 1
4- Others: No = 0 Yes = 1 (specify)…….

F. ORAL AND DENTAL

1- Dental malocclusion: No = 0 Yes = 1 N/A = 2
2- High arched palate: No = 0 Yes = 1
3- Articulation difficulties: No = 0 Yes = 1 N/A = 2
4- Giant cell lesions: No = 0 Yes = 1
5- Others: No = 0 Yes = 1 (specify)……...

G. CARDIOVASCULAR

1- **Congenital heart defect(s):** PS = 1 ASD = 2 VSD = 3 AVSD = 4
PDA = 5 Mitral valve defect = 6 tetralogy of fallot = 7
Others (specify)……. = 8
2- **Hypertrophic cardiomyopathy**: No = 0 Yes = 1
3- **Electrocardiographic abnormalities**: left axis deviation = 1 negative aVF = 2
Abnormal R/S ratio over the left precordium = 3 abnormal Q wave = 4
4- **Others**: No = 0 Yes = 1 (specify)……...

H. MUSCULOSKELETAL

1- **Chest**: pectus carinatum superiorly & pectus excavatum inferiorly = 1
Pectus carinatum = 2 pectus excavatum 3 none = 4
2- **Spine**: scoliosis = 1 other anomalies (specify)……. = 2 none = 3
3- **Limbs**: Cubitus valgus = 1 Talipes equinovarus = 2 others……...…. = 3
4- **Joints**: contracture = 1 radio-ulnarsynostosis = 2 cervical spine fusion = 3
Hyperextensibility = 4 none = 5
5- **Others**: No = 0 Yes = 1 (specify)……

I. GASTROINTESTINAL

1- Feeding difficulties: poor suck with prolonged feeding time = 1 Very poor suck and slow feeding with recurrent vomiting = 2 severe feeding problems that require tube-feeding for ≥ weeks = 3
2- Hepatomegaly in infancy: No = 0 Yes = 1 unknown = 2
3- Others: No = 0 Yes = 1 (specify)……

J. GENITOURINARY

1- Undescended testis: No = 0 Yes = 1 N/A = 2

2- Renal abnormalities: No = 0 Yes = 1
If Yes specify……..
3- Sertoli cells dysfunction: No = 0 Yes = 1 Unknown = 2
4- Others: No = 0 Yes = 1 (specify)……

K. DERMATOLOGY

1- Thick curly hair: No = 0 Yes = 1
2- Pigmented naevi: No = 0 Yes = 1
3- Café au lait spots: No = 0 Yes = 1
4- Keratosis pilaris atrophicans faciei: No = 0 Yes = 1
5- Others: No = 0 Yes = 1 (specify)……

L. HAEMATOLOGY AND ONCOLOGY

1- Bleeding diathesis: Easy bruising: No = 0 Yes = 1
2- Intrinsic pathway abnormalities: Factor VIII = 1 Factor XI = 2 Factor XII = 3 others (specify)…….= 4 none = 5 Unknown = 6
3- Thrombocytopenia: No = 0 Yes = 1
4- Platelet function abnormalities: No = 0 Yes = 1Unknown = 3
5- Transient myeloproliferative disorder: No = 0 Yes = 1
6- Leukaemia: No = 0 Yes = 1 (specify)……..
7- Solid tumours: No = 0 Yes = 1 (specify)……...
8- Others: No = 0 Yes = 1 (specify)…….

M. LYMPHATIC

1- Lymphedema: No = 0 Yes = 1
2- Lymphangiectasia: No = 0 Yes = 1
3- Chylous effusion: No = 0 Yes = 1
4- Others: No = 0 Yes = 1 (specify)……

N. NEUROLOGY, EDUCATION AND BEHAVIOUR

1- Mild intellectual disability: No = 0 Yes = 1
2- Learning difficulties: No = 0 Yes = 1N/A = 2
3- Recurrent seizures: No = 0 Yes = 1
4- Behavioural issues: No = 0 Yes = 1(specify)……..
5- Others: No = 0 Yes = 1 (specify)…….

Appendix 2: English version

UNIVERSITY OF CAPE TOWN
DIVISION OF HUMAN GENETICS

INFORMATION SHEET FOR PARTICIPANTS OF RESEARCH STUDY:

"Genetic investigation of South Africans with the Noonan Syndrome Phenotype using Targeted Next Generation Sequencing"

Introduction

You have been approached to participate in this study because you or your family member has been diagnosed with probably Noonan syndrome.

People with Noonan syndrome have variable characteristics but do tend to share similar features such as facial appearance and short stature while still developing. Most adults with Noonan syndrome are of normal height. Some people with Noonan syndrome may have other problems, such as heart problems or having difficulties with learning at school. However, the majority of people with Noonan syndrome can have a relatively normal life if they are identified early and have regular monitoring for any problems that may arise.
Noonan syndrome is an autosomal dominant disorder. This means that boys and girls can be affected with equal chance. Often the individual diagnosed with Noonan syndrome is the first and only individual that is affected in a family, however, once it is in the family it can be passed on and there are families where many people are affected and have Noonan syndrome. However, even in the same family, some people can have more severe symptoms than others and sometimes some people do not have any symptoms at all. Noonan Syndrome is caused by a change in one of our genes (a piece of DNA), which are the instructions to make a person. If a father or mother carries a change or mistake in one of the genes that cause Noonan syndrome, they will have a 50% chance of passing it on to their son or daughter, causing him/her to have Noonan syndrome

Why are we doing this study?

The aim of this study is to identify individuals who have a likely diagnosis of Noonan syndrome and perform genetic testing to identify the genetic mistake which has caused this condition in an individual. The hope is that this will enable us to confirm if you are really affected or not and to offer better testing in the future for other families affected by Noonan's syndrome. The researchers also hope to determine if there are any specific genetic changes that cause specific features of the condition or not.

What will you ask me to do if I agree to participate in this study?

If you agree to be a part of this study, you will be clinically examined by the doctor performing this research. In addition, a blood sample will also be taken.

What will happen to my samples and information?

Your/your child's blood will be analysed to look for changes in the DNA that would explain why they have Noonan syndrome. The DNA will be stored for future possible testing. This information may be used to produce publications but no identifiable data will be used.

Confidentiality

All information given is confidential. All the information gathered during this study will be safely stored in locked offices or in password protected computers. The DNA will be coded and the individual working on the sample will not know who it belongs to. The researcher will not discuss your participation in the research with any other patients or individuals not involved with your medical care. You may be asked to give permission/consent to publish photos if these are taken during the clinical examination but this will be your decision.

What are the risks of being involved in the study?

The risk of harm or discomfort expected during this study is not greater than those you encounter in daily life or during routine medical examinations. The prick of the needle during blood taking carries the same risks as having bloods taken for any other reason. There may be some bruising but this is not usually a serious problem.

Are there any benefits to me?

The research may identify the cause of Noonan syndrome in the family, potentially confirming your/your child's diagnosis of Noonan syndrome. Should something be identified, all effort will be made to contact you and explain the result to you in person. This will provide you with information which can be helpful for you/your child and your family. If a change/mistake is identified, the family may also be offered genetic testing if they are interested. Please do inform us if your contact details change during the study period so that you may be contacted if needed.

Do I have to participate?

No you do not, this is entirely voluntary. If at any time during the study you wish to withdraw your consent you may do so without any harm to your medical care. You will be treated the same whether you participate in the study or not. Please feel free to ask your family members or close friends opinion's if you need advice before agreeing to participate to the study.

Please feel free to contact the following people if you have any problems or questions about the research:

Dr Cedrik Ngongang
Tel: 021 406 6698
E-mail: Cedrik.Ngongang@uct.ac.za

Prof Ambroise Wonkam
Tel: 021 406 6307
E-mail: Ambroise.Wonkam@uct.ac.za

SrSolomons
Tel: 021 406 6304

If you have any questions regarding your rights as participants in this research project please contact Prof M. Blockman, Chair of the Research Ethics Committee of the University of Cape Town on 021 406 6496.

Appendix 3: English version

UNIVERSITY OF CAPE TOWN
DIVISION OF HUMAN GENETICS

INFORMED CONSENT FOR RESEARCH PROJECT:

"Genetic investigation of South Africans with the Noonan Syndrome Phenotype using Targeted Next Generation Sequencing"

I, ……., hereby voluntarily consent to my child …….. / My genetic material being investigated for a disease causing mutation in the genes causing Noonan syndrome in my family.

INFORMATION REGARDING RESEARCH STUDY:

The objectives of this study are:

1. To identify, families and individual patients with Noonan syndrome;
2. To undertake detailed clinical examination of each selected patient;
3. To perform molecular analysis on a genetic sample from each patient;
4. To establish the Genotype-Phenotype correlation of Noonan syndrome within the study population

Each participant will have:

1. A clinical examination done by one of the medical doctors at Groote Schuur Hospital or Red Cross War Memorial Children Hospital
2. A blood sample taken to extract DNA for genetic studies

DNA of people suspected to be affected will be analysed only for mutations in genes causing Noonan syndrome. If any mutation is found, at risk family members DNA may be tested in the future for the same mutation. By consenting for this study you are also consenting for your DNA to be stored for future use. Any further genetic investigations will relate only to Noonan syndrome.
All results will be delivered to the relevant persons at the end of the study period where possible. Implications of these results will also be discussed in detail as part of the genetic counselling process.

By consenting to this research study permission is also granted for publishing of the results.
No identifiable data will be used in publication although photos may be published if your consent is given. Everything is confidential

Participation in this study is voluntary and may be withdrawn at any stage, without any harm to future care.

Signature: …… Date: ……

Witness signature: …….. Date: ……

Appendix 4: English version

UNIVERSITY OF CAPE TOWN
DIVISION OF HUMAN GENETICS

ASSENT FOR RESEARCH PROJECT:

"Genetic investigation of South Africans with the Noonan Syndrome Phenotype using Targeted Next Generation Sequencing"

1. This study is being done by the doctors at the University of Cape Town to try and learn more about a genetic condition called Noonan syndrome.
2. You and some members of your family have been asked to participate in the study because Noonan syndrome may run in your family.
3. The doctors are trying to see if they can learn more about how someone with Noonan syndrome looks, and how it happens. This is so they can better help you and other people's families in the future.
4. If you decide to be a part of the study one of the doctors from Genetics will examine you, and also collect some blood from you.
5. Nothing that will be done will be harmful to you.
6. Everything that is done during the study will be confidential, meaning that no one who is not a part of the study will know that you participated.
7. You can decide for yourself whether you want to be a part of this study. If you decide to not be a part of it then no one will be angry with you, and you will still get all the help and care that you need.

Signature: …… Date: ……..

Witness signature: …… Date: ……

Appendix 5: Xhosa version

UNIVESITHI YASE KAPA
KWICANDELO LWEZOFUZO LOLUNTU
IPHEPHA ELINOLWAZI LWABANTU ABAZOBAYINXALANYE YOPHANDO:

"UphandolwezofuzokuluntulwaseMzantsiAfrikaabaphilanesimo with seNoonan Syndrome"

Igama lam nguCedrik Ngongang, ugqirha wezofuzo ekuqeqesheni kwiYunivesithi yaseKapa, kwiCandelo loLuntu lweZofuzo.

Uye watyelelwa ukuba uthathe inxaxheba kwe sisifundo ngenxa yokuba wena okanye ilungu lentsapho yakho sele lufumene i-Noonan syndrome.

Abantu abane-Noonan syndrome baneempawu ezichaseneyo kodwa bathambekele ukwabelana ngezinto ezifana nokubonakala kwesobuso kunye nokuba mfutshane ngokwesiqu ngelixa besakhulayo. Uninzi lwabantu abadala abaneNoonan syndrome luye lubenesiqu esiqhelekileyongokobude. Abanye abantu abanesifo seNoonan syndrome banokuba nezinye iingxaki, ezifana neengxaki zentliziyo okanye ubunzima bokufunda esikolweni. Nangona kunjalo, uninzi lwabantu abane-Noonan syndrome banokuba nobomi obuqhelekileyo ukuba babonwa ngokukhawuleza kwaye bajongwe rhoqo naziphi na iingxaki ezinokuvela.
I-Noonan syndrome yiAutosomal dominant disorder. Oku kuthetha ukuba abafana kunye namantombazana banokuchaphazeleka ngamathuba alinganayo. Ngokuqhelekileyo umntu ofunyanwe yi-Noonan syndrome ngumntu wokuqala kunye nomntu wokuphela ochaphazelekayo kwintsapho, nangona kunjalo, xa ikwintsapho ingadluliselwa kwaye kukho iintsapho apho abantu abaninzi banokuchaphazeleka sesisigulo. Nangona kunjalo, kwintsapho enye, abanye abantu banokuba nezibonakaliso ezinzima kunabanye kwaye ngamanye amaxesha abanye abantu abanayo nayiphi na impawu. I-Noonan Syndrome ibangelwa lutshintsho kwelinye lezityalo zethu (isicatshulwa seDNA), eziyimigaqo yokwenza umntu. Ukuba utata okanye umama unotshintsho okanye impazamo kwenye yezinto eziphilayo ezenza i-Noonan syndrome, baya kuba nethuba elingama-50% lokuyidlulisela kwindodana okanye intombi yabo, kumenze ukuba abe ne-Noonan syndrome.

Kutheni sisenza esi sifundo?

Injongoyesisifundo kukufumanisa abantu abanothi babenamathuba okufumana i-Noonan syndrome kwaye kwenziwe iimvavanyo zofuzo ukuze bachazwe impazamo yeDNA ezibangele le meko kumntu. Ithemba kukuba oku kuya kusinceda ukuba siqinisekise ukuba uchaphazelekile okanye awuchaphazelekanga, okunye kukunika uvavanyo olungcono kwixesha elizayo kwezinye iintsapho ezichaphazelekayo yi-Noonan syndrome. Abaphandi banethemba lokuqinisekisa ukuba alukho na olunye utshintsho kwimfuzo eyenza impawu ezithile zemo okanye hayi.

Yintoni oya kundicela ukuba ndiyenze ukuba ndiyavuma ukuthathainxaxheba kwesisifundo?

Ukuba uyavuma ukuba yinxalenye yesisifundo, uya kuvavanywa ngugqirha owenza oluphando. Ukongezelela, uzotsalwa intwana yegazi.

Kuzokwenzekani kwi gazi lam nolwazi ngam?

Igazi lakho okanyeelomntwana wakho liya kujongisiswa ukubona utshintsho kwi-DNA echaza isizathu sokuba babenesifo se-Noonan Syndrome. I-DNA iya kugcinelwa ukuvavanywa kwe xesha elizayo. Le ngcaciso ingasetyenziselwa ukuvelisa iimpapasho kodwa akukho lwazi oluchanekileyo ngawe elinothi lusetyenziswe.

Imfihlelo

Yonke inkcazelo enikeziweyo iyimfihlelo. Yonke ingcaciso nolwazi olubuthwe ngexesha lesisifundo iya kugcinwa ngokukhuselekileyo kwiiofisi ezivaliweyo okanye kwiikhompyutha ezikhuselweyo zegama. I-DNA iya kubhalwa ngendlela efihlakeleyo kwaye umntu osebenza kweyakho akayi kukwazi ukuba ungubani. Umphandi akayi kubalisa ngenxaxheba yakho koluphando kunye naziphi na izigulane okanye abantu abangabandakanyekanga kunyango lwenu. Unokucelwa ukuba unike imvume yokupapasha iifoto ukuba ziye zathathwa ngexesha loviwo lweklinikhi kodwa oku kuya kuba sisigqibo sakho.

Ziziphi iingozi zokubandakanyeka kwisifundo?

Umngcipheko wokulimala okanye ukungaxhamli okulindeleke kwesisifundo akwethusi. Xa kuthathwa igazi ngenaliti kuyafana naxa utsalwa igazi kwezinye izigulo. Ungadumba kancinci kodwa akafani kubeyinto exhalabisayo.

Ingaba zikhona naziphi na iinzuzokum?

Umphandi angakuchazela unobangela wesisigulo kusapho lwakho.Ukuba kuthe kwachaneka nto uzobizwa uchazelwe iziphumo. Oku kungakunikeza ulwazi olunoku nceda wena nosapho lwakho. Ukuba kufumaniseke utshintsho kwi DNA yakho, usapho lonke lungavavanywa ukuba lunomdla. Sicela usazise ukuba inombolo zakho zefowuni ziye zatshintsha ngelixesha lesisifundo ukwenzela sikwazi ukukufumana xa kudingeka.

Kunyanzelekile ukuba ndithathe inxaxheba?

Hayi akunyanzelekanga, yonke lento iphuma kuwe. Ukuba uthe wafuna ukurhoxa sesiqhuba esisifundo unalo ilungelo lokurhoxa kwaye lonto ayichaphazeli impilo yakho. Izigulane zonke zizophathwa ngokulinganayo noba awuyo nxalenye yesisifundo. Ukhululkile ukuba ungacela ingcebiso kwizihlobo nakusapho lwakho phambi kokuba uvume ukuba koluphando.

Ukhululekile ukuba ungafowunela ababantu bangezantsi ukuba uthe waba nemibuzo okanye iingxaki ngoluphando:

Dr Cedrik Ngongang
Tel: 021 406 6698
E-mail: Cedrik.Ngongang@uct.ac.za

Prof Ambroise Wonkam
Tel: 021 406 6307
E-mail: Ambroise.Wonkam@uct.ac.za

Sr Solomons
Tel: 021 406 6304

Ukubaunemibuzomayelananamalungeloakhonjengomntuothathainxaxhebakoluphandosicela ufowunele u Prof M. Blockman, usihlalowekomitiyezophandokwiUnivesithiyaseKapakulenombolo 021 406 6496

Appendix 6: Xhosa version

UNIVESITHI YASE KAPA
CANDELO LWEZOFUZO LOLUNTU
ULWAZI LWEMVUME YAKHO KOLUPHANDO:

"UphandolwezofuzokuluntulwaseMzantsiAfrikaabaphilanesimoseNoonan Syndrome"

Mna……ndiyavumanokuzinikezelayoukubaumntwanawam…….. Okanyemnasingasetyenziswakoluphandolwezofuzokwisiguloesiyi Noonan Syndrome kusapholwam.

ULWAZI MAYELANA NOLUPHANDO:

Iinjongozoluphando:

1. Kukuchazaamasaphokunyenabantuabagulabodwayi Noonan Syndrome;
2. Ukwenzauviwooluphelele yolweklinikhikwisigulanengasinyeesikhethiweyo;
3. Ukujongisisaimolecularsampulikwisigulanengasinye;
4. Ukufumanisauhambelwanophakathikwendlelaoyiyongaphakathinendlelaobanakalangayongaphandlephakathikoluntuokuphandonalo.

Umntungamnyeokuphandouzoba:

1. NovavanyolwaseklinikhielizokwenziwangomnyewogqirhakwisibhedleleiGrooteSchuurokanyeisibhedleleiRed Cross War Memorial Children Hospital.
2. Nethyubhuengu 2-5ml yegazielizotsalwaukuzekufunyanweiDNAezosetyenziswakoluphandolwezofuzo.

IDNA yabantuabakrokrelekaukucaphazelekasesisifoizovavanywakukhangelweutshintshoelubangelaesisifo.Ukubakuthekwafumanekautshintshokumalunguosaphoasengoziniyokusifumanaesisigulo,iDNAyaboingaphindayenziweimviwokwixeshaelizayo. NgokuvumaoluphandookanyeisifundouvumanokubaiDNAyakhoigcinelweukusetyenziswakwilixaelizayo.Uphandolwezofuzoelinothiliphindelwenziwelizokubalele Noonan Syndrome kuphela.Zonkeiziphumozizonikwaumntuofanelekileyoekuphelenikoluphandoaphokwenzekakhona.Impembelelo yale miphumo iya kuxoxwa ngokubanzi njengengxenye yenkqubo

yokucebisa ngezofuzo.
Ngokuvumakulemvumeyokufundauphandokwakhonakunikezaimvumeyokupapashwakwezip humo.Akukholwazingaweoluchanekileyoelizakusetyenziswaekupapashweninangonaiifotozin okupapashwaukubaimvumeyakhouyinikezile.Yonke into iyimfihlo.
Ukubayinxalenyeyoluphandoakunyanzelekangakwayeungarhoxanannininaufuna. Oku akungekhekuchaphazeleimpiloyakho.
Sayina: …….. Umhla: ……
Umsayino wengqina: ..... Umhla: .....

Appendix 7: Xhosa version

UNIVESITHI YASE KAPA

CANDELO LWEZOFUZO LOLUNTU

IMVUMI YOPHANDO:

"Uphando lwezofuzo kuluntu lwase Mzantsi Afrika abaphila nesimo seNoonan Syndrome"

1. Oluphando lwenziwa ngogqirha base Univesithi yase Kapa ukuzama ukufunda kabanzi ngesimo sezofuzo iNoonan Syndrome.
2. Wena namanye amalungu osapho lwakho nicelwe ukuba nibe yinxalenye yesisifundo ngokuba esisifo singachaphazela usapho lwakho.
3. Oogqirha bazama ukufunda kabanzi ukuba umntu onesisigulo ubukeka njani nokuba senzeka njani. Okukulungiselelaukuncedawenanamanyeamasaphokwilixaeliphambili.
4. Ukubauthewavumaukubayinxalenyeyoluphando, omnyewogqirhabazokuvavanyakwayebatsaleithyubhuyegaziengu 2-5ml.
5. Akhontoenobungoziezokwenzekakuwe.
6. Yonke into ezokwenziwakwesisifundoizobayimfihlelo. Okokuthethaukuthiumntuongeyonxalenyenesisifundoakangekheayaziukubawenautha theinxaxheba.
7. Uyakwaziukuzikhethelaukubayinxalenyeyesisifundoawunyanzelekanga. Ukubautheawavumaakekhoumntuozokulwisa, usezolufumanauncedooludingayo.

Sayina……. Umhla ……

Umsayinowengqina…….Umhla……

Appendix 8: Afrikaans version

UNIVERSITEIT VAN KAAPSTAD
DIVISIE VAN MENS GENETIKA
INFORMASIEBLAD VIR DEELNEMERS AAN NAVORSINGSTUDIE:

"Genetiese ondersoek van Suid-Afrikaners met die Noonan sindroom fenotipe, deurmiddel van volgende generasie volgorde bepaling (Targeted Next Generation Sequencing)"

Geagte leser,

My naam is Cedrik Ngongang en ek is n mediese genetika dokter tans in opleiding by die Universiteit van Kaapstad, Divisie van Mens Genetika.

U is genader om deel te neem aan hierdie studie omdat u of 'n familielid, potensieel met Noonan Sindroom gediagnoseer is.

Mense met Noonan sindroom het 'n wye reeks van eienskappe, maar mees erkenbaar is 'n karakteristieke gesigs voorkoms en die geneigheid om korter as normaal te wees. Die meeste volwassenes met Noonan sindroom is egter normale lengte. Sommige mense met Noonan Sindroom mag ook ander problem hê soos hart- of leerprobleme. Ten spyte hiervan sien ons dat die meeste mense met Noonan sindroom'n relatiewe normale lewe lei. Vroeë diagnose en gereelde mediese opvolg is belangrik om moontlike probleme te identifiseer. Noonan sindroom is 'n outosomaal dominante kondisie. Dit beteken dat seuns en dogters 'n gelyke kans het om geaffekteer te wees. Ons sien ook dikwels dat 'n persoon wat met Noonan sindroom gediagnoseer word, die eerste en enigste geaffekteerde persoon in die spesifieke familie is. Sodra die sindroom egter in 'n familie voorkom, kan dit oorgeërf word en dus is daar families waar verskeie lede Noonan sindroom het. In een familie sien ons egter wel 'n spektum van simptome, dit beteken dat sekere lede ernstige simptome kan toon terwyl ander geen simptome toon nie.

Noonan sindroom word veroorsaak deur ‘n verandering in een van ons gene. DNA bestaan uit gene wat die instruksies in ons liggame is om proteine te maak. Dit kan ook gesien word as elke individue se spesifieke bouplan. Indien ‘n vader of moeder ‘n verandering (of fout) dra in een van die gene wat Noonan sindroom veroorsaak, is daar dus met elke kind ‘n 50% kans dat dit aan hulle oorgedra kan word. ‘n Kind met die oorgeërfde verandering sal dan Noonan sindroom hê.

Waarom doen ons hierdie studie?

Die doel van hierdie studie is om individue wat ‘n moontlike diagnose van Noonan sindroom het, te identifiseer en genetiese toetse op hulle te doen. Sodoende kan ons moontlik die genetiese fout identifiseer wat die kondisie veroorsaak het. Ons hoop dus om in die toekoms mense meer akkuraat te kan diagnoseer en om beter toetse te kan bied vir families wat deur Noonan sindroom geaffekteer word. Verdere navorsing rakende Noonan sindroom kan ook moontlik navorsers in staat stel om te bepaal of daar enige spesifieke genetiese veranderinge is wat spesifieke kenmerke van die Noonan sindroom veroorsaak.

Wat sal van my gevra word as ek instem om deel uit te maak van hierdie studie?

Indien u instem om deel te neem aan hierdie spesifieke studie, sal u ondersoek word deur die dokter en ‘n bloed monster sal by u geneem word.

Wat sal gebeur met die informasie verkry uit die ondersoek van my bloedmonster?

U/u kind se bloed sal geanaliseer word om te kyk vir veranderinge in die DNA wat moontlik kan verduidelik waarom u/u kind Noonan sindroom het. Die DNA sal gestoor word vir moontlike toekomstige toetse. Hierdie informasie mag gebruik word vir publikasies, maar geen identifiserende besonderhede sal bekend gemaak word nie.

Vertroulikheid

Alle informasie verskaf sal as vertroulik gesien word. Alle inligting wat deur hierdie studie ingesamel word, sal veilig gestoor word in geslote kantore of in wagwoord-beskermde rekenaars. Die DNA sal gekodeer word en die individu wat met die monster werk sal nie weet aan wie dit behoort nie. Die navorser sal nie u deelname aan die studie bespreek met enige ander pasiënte of persone wat nie by u mediese sorg betrokke is nie. Indien daar foto’s geneem word tydens u kliniese ondersoek, mag u dalk gevra word om skriftelike toestemming te verleen om die foto’s te publiseer. Dit sal egter u keuse wees of u toestemming wil verleen of nie.

Wat is die risiko's van deelname aan hierdiestudie?

Die risiko vir skade of ongemak wat tydens hierdie studie verwag word, is nie groter as dit wat u in die daaglikse lewe of gedurende 'n roetine mediese ondersoek ervaar nie. Die naald prik tydends die neem van u bloed dra dieselfde risiko's as om bloed vir enige ander rede te neem. Daar mag dalk kneusing wees, maar dit is gewoonlik nie 'n ernstige probleem nie.

Is daar enige voordele vir my?

Die studie mag die oorsaak van Noonan sindroom in u familie identifiseer. Potensieel kan 'n diagnose van Noonan sindroom in u/u kind bevestig word. Indien 'n oorsaak of variant geïdentifiseer word, sal alle pogings gemaak word om u te kontak en die resultate in persoon met u te bespreek. Informasie wat nuttig mag wees vir u/u kind en u familie sal verskaf word. Indien 'n verandering/fout geïdentifiseer word, mag die res van die familie genetiese getoets word, as hulle sou belangstel en instem. Laat ons asseblief weet, indien u kontak besonderhede sou verander gedurende hierdie studie periode, sodat ons u kan bereik indien nodig.

Moet ek aan die navorsing deelneem?

Nee, dit is u eie keuse. U deelname aan hierdie studie is heeltemal vrywillig. Dit is ook moontlik om enige tyd tydens die studie u deelname te beëindig en u mediese sorg sal nie nadelig beinvloed word nie. U sal dieselfde mediese behandeling ontvang of u instem om deel te neem aan die studie of nie. Neem asseblief die vrymoedigheid om u familie en naaste vriende se opinie te vra indien u onseker is oor deelname aan hierdie studie.
U kan enige een van die volgende mense kontak indien u enige probleme of verdere vrae rakende die navorsing het:

Dr Cedrik Ngongang
Tel: 021 406 6304
E-mail: Cedrik.Ngongang@uct.ac.za

Prof Ambroise Wonkam
Tel: 021 406 6307
Email: Ambroise.Wonkam@uct.ac.za

Sr Solomons
Tel: 021 406 6304

Indien u enige vrae rondom u regte as deelnemer aan hierdie navorsing studie het, kan u Prof. M Blockman, hoof van die Navorsing Etiese Komitee (Research Ethics Committee) van die Universiteit van Kaapstad kontak by 021 406 6496.

Appendix 9: Afrikaans version

UNIVERSITEIT VAN KAAPSTAD
DIVISIE VAN MENS GENETIKA
INGELIGTE TOESTEMMING TOT NAVORSINGSPROJEK:

"Genetiese ondersoek van Suid-Afrikaners met die Noonan sindroom fenotipe, deurmiddel van volgende generasie volgorde bepaling (Targeted Next Generation Sequencing)"

Ek, ……,gee hiermee vrywilliglik toestemming dat my kind…….... /my genetiese material ondersoek mag word vir moontlike siekte-veroorsakende mutasies in die gene wat Noonan sindroom in my familie veroorsaak.

INFORMASIE RAKENDE NAVORSINGSTUDIE :

Die doelstelling van hierdie studie is :

1. Om individue en families met Noonan sindroom te identifiseer
2. Om 'n volledige kliniese ondersoek van elke geselekteerde pasiënt te doen
3. Om molekulêre analise op die genetiese monster van elke pasiënt te doen
4. Om die genotipe-fenotipe korrelasie van Noonan sindroom in die studie-populasie vas te stel

Elke deelnemer sal:

1. 'n Kliniese ondersoek deureen van die dokters by die Groote Schuur Hospitaal of Rooikruis Kinderhospitaal ondergaan
2. **Een buisie van 2-5ml bloed** sal geneem word om DNA te verkry vir verdere genetiese studies

Die DNA van die mense wat vermoedelik geaffekteer mag wees, sal slegs geanaliseer word vir moontlike mutasies in gene wat Noonan sindroom veroorsaak. Indien enige mutasie gevind word, mag familielede wat die risiko dra om dieselfde gene te hê in die toekoms getoets word vir dieselfde mutasie. Deur in te stem tot hierdie studie, stem u ook in dat u DNA geberg mag word vir toekomstige gebruik. Enige toekomstige genetiese ondersoeke sal slegs verband hou met Noonan sindroom.

Alle resultate sal, waar moontlik, gelewer word aan die relevante persone aan die einde van die studieperiode. Implikasies vir hierdie resultate sal ook bespreek word as deel van die genetiese raadgewings proses.

Deur in te stem tot hierdie navorsing studie word toestemming ook gegee vir die publikasie van die resultate. Geen identifiserende besonderhede sal in die publikasie gebruikword nie, maar foto's mag wel gepubliseer word indien u toestemming daarvoor gegee het. Alle informasie is konfidensieel.

Deelname aan hierdie studie is vrywillig en u mag op enige stadium onttrek sonder om enige toekomstige sorg te benadeel.

Handtekening: …… Datum: ……

Getuiehandtekening: ……..……. Datum: ……

Appendix 10: Afrikaans version

UNIVERSITEIT VAN KAAPSTAD

DIVISIE VAN MENS GENETIKA

TOESTEMMING VIR NAVORSINGSPROJEK:

"Genetiese ondersoek van Suid-Afrikaners met die Noonan sindroom fenotipe, deurmiddel van volgende generasie volgorde bepaling (Targeted Next Generation Sequencing)"

1. Hierdie studie word gedoen deur die dokters by die Universiteit van Kaapstad en poog om meer te leer oor 'n genetiese kondisie genaamd Noonan sindroom.

2. U en sekere van u familielede is gevra om deel te neem aan die studie aangesien Noonan sindroom moontlik in u familie mag voorkom.

3. Die dokters probeer om meer te leer oor hoe iemand met Noonan sindroom lyk en hoekom die kondisie gebeur. Hiermee poog hulle om u en ander mense se families in die toekoms better te kan help.

4. Indien u besluit om deel van die studie te wees, sal een van die Genetika dokters u ondersoek en **een buisie bloed van 2-5ml** neem.

5. Niks wat tydens die studie gedoen word sal u skade berokken nie.

6. Alle informasie wat gedurende die studie verkry word is vertroulik, dit beteken dat niemand wat nie deel van die studie is nie, sal weet dat u deelgeneem het nie.

7. U kan self besluit of u deel van hierdie studie wil wees. Indien u besluit om nie deel te neem nie sal niemand vir u kwaad wees nie en u sal steeds al die hulp en sorg ontvangs wat u benodig.

Handtekening: …… Datum: ……

Getuiehandtekening: ……..…… Datum: ……

Appendix 11: Ethics Approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

31 October 2016

HREC REF: 449/2016

Prof A Wonkam
Human Genetics
Suite N3
Werner & Beit North Building

Dear Prof Wonkam

PROJECT TITLE: GENETIC INVESTIGATION OF SOUTH AFRICANS WITH THE NOONAN SYNDROME PHENOTYPE USING TARGETED NEXT GENERATION SEQUENCING (MMeD-candidate-Dr C Tekendo)

Thank you for your letter to the Faculty of Health Sciences Human Research Ethics Committee dated 8 September 2016.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th October 2017.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period. (Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

The HREC acknowledge that the student Dr Cedrik Ngongang Tekendo will also be involved in this study.

Yours sincerely

signature removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC 449/2016

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.

The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.