



A clinical and molecular investigation of two families with Simpson-Golabi-Behmel syndrome

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1 PROTOCOL

1.1 INTRODUCTION

Clinical features

Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked skeletal overgrowth disorder. The exact prevalence of SGBS is unknown, but it is believed to be under-diagnosed¹. By 2005 at least 130 patients had been reported on worldwide².

This disorder is characterised by pre- and postnatal macrosomia. Facial features are described as being “coarse”. Distinctive facial features are those of an upturned nose, hypertelorism and downslanting palpebral fissures. Other manifestations such as macrostomia, macroglossia and a midline groove in the lower lip or tongue are often present. Less commonly cleft lip and palate can also occur. Supernumerary nipples are frequent. Short hands and feet with poly- or syndactyly have also been reported³.

Structural abnormalities include umbilical and diaphragmatic hernias as well as genitourinary defects³. Cardiac abnormalities are present in 36% of patients with SGBS⁴. Skeletal abnormalities include vertebral fusion, scoliosis, pectus excavatum and rib anomalies¹. Development can be normal, or mildly delayed³.

Persons with SGBS have a 10% risk of developing embryonal tumours, and tumour surveillance is necessary. The tumours described in SGBS are Wilms tumour, hepatoblastoma, adrenal neuroblastoma, gonadal blastoma and hepatocellular carcinoma⁵.

Carrier females can have mild manifestations of SGBS due to skewed X-inactivation⁶. Other possible reasons for females manifesting signs of SGBS are numerical X-chromosome abnormalities (e.g. Turner syndrome), X-autosome translocations, and the presence of mutations on both X-chromosomes. The features of SGBS can range from minimal, such as a large mouth and coarse facies with normal intelligence; to more severe signs such as developmental delay, cardiac abnormalities, and macrosomia⁶.

Medical genetics and genetic counselling

SGBS is an X-linked recessive disorder and males with the mutation will manifest the phenotype, whereas women with the mutation are termed “carriers”, and are usually clinically unaffected. When a woman carrying this mutation has a child, she would have a 50% chance of passing the abnormal X-chromosome in each pregnancy. She would thus have a 25% chance of having an affected son, and a 25% chance of having a carrier daughter.

Molecular genetics

Mutations in two genes, glypican 3 (*GPC3*) and the adjacent glypican 4 (*GPC4*) have been reported to be responsible for SGBS.

The *GPC3* gene at Xq26 spans 500kb of genomic DNA and consists of eight exons. *GPC3* encodes glypican 3, a cell surface heparan sulfate proteoglycan, that plays a role in cell growth and division. Mutations in *GPC3* that cause SGBS can be deletions or point mutations, both of which cause loss of function of the *GPC3* product. There are no known hotspots for mutations. Most point mutations occur in exon 3 (the largest exon), and 50% of deletions occur in exon 8⁷. Results of studies investigating the detection rate of *GPC3* mutation in patients with SGBS vary greatly. Some studies report detection rates of 37%⁸, whereas others have rates as high as 70%⁷.

The *GPC4* gene, also at Xq26, is adjacent to the 3' end of *GPC3*. Previously, a deletion involving the 3' end of *GPC3* and spanning *GPC4* was described in a patient with SGBS⁷. Loss of function of *GPC4*, without involvement of *GPC3* has not been reported to cause SGBS. However, recently a duplication of *GPC4* has been found in a family with SGBS⁹. For the purpose of this study, *GPC4* will not be investigated.

1.2 JUSTIFICATION

No studies from South Africa relating to SGBS could be found during a Pubmed, and Google scholar search. Key words used were “Simpson-Golabi-Behmel syndrome and South Africa”.

Diagnostic testing for SGBS is not currently available in South Africa. The diagnosis of SGBS is made on clinical examination, unless a disease causing mutation can be identified.

Careful review of medical records and examination of the two affected probands will help by identifying frequent clinical features, in turn this will facilitate diagnosis in the future as it is generally accepted that SGBS is under-diagnosed. Careful delineation and comparison of a clinical phenotype to reports in the literature would also help with diagnosis of affected persons in the future.

Analysis and sequencing of *GPC3* has the potential to confirm a clinical diagnosis. If a disease causing mutation is found, it would be appropriate to go on to determine whether or not the affected male’s mother is a gene mutation carrier. Knowing the mother’s carrier status will firstly clarify her risk of having another affected son or carrier daughter. Secondly, it will have implications for the family as other members could potentially be mutation carriers and at risk of having a boy with SGBS.

Establishing the technology for SGBS testing in this study could potentially result in future diagnostic testing for South Africa, if it is found to be cost effective.

1.3 AIM

The identification of a disease causing mutation in two families with a clinical diagnosis of Simpson-Golabi-Behmel syndrome.

1.4 OBJECTIVES

The objectives of this study will be as follows:

1. Describing the phenotype of two male probands, with a clinical diagnosis of Simpson-Golabi-Behmel syndrome, and their family members.
2. Molecular analysis of the *GPC3* gene using DNA from the affected probands in an attempt to isolate a disease-causing mutation.

1.5 METHODS

1.5.1 Study design

This study is a **case series** with a clinical and a molecular component

1.5.2 Subject identification

Two male probands in different families (proband B and S) seen by the Division of Human Genetics, University of Cape Town were identified following a diagnosis of SGBS made during clinical evaluation. These two young boys will form the starting point for this study. The two boys and their respective mothers will be clinically re-examined and examined in conjunction with members of the Genetics team. Proband S has a maternal aunt reported to have intellectual difficulties and she, as well as his maternal grandmother will also be examined if possible. This approach will be undertaken as both these women are at risk of being carriers, and could manifest some signs of SGBS.

1.5.3 Recruitment and Enrolment

Proband B was identified when a referral was made to the Division of Human Genetics from Groote Schuur Hospital. Proband S was seen at the Genetic Clinic at Red Cross War Memorial Children's Hospital as an outpatient.

During a previous consultation the mother of proband B was informed of the possibility of genetic testing to confirm a diagnosis in her son, in which she expressed a keen interest.

Both probands' mothers will be personally informed regarding the research project during a scheduled visit to the Division of Human Genetics.

Both mothers will be invited to participate in the study. The mother of proband S will be asked to invite her sister and mother to the information giving session. These family members will also be invited to participate in the research study.

Their participation will be voluntary and subject to informed consent.

1.5.4 Measurements and methods

Inclusion criteria for patients into the study:

Boys clinically affected with SGBS

Mothers of affected boys

Family members with intellectual or medical difficulties related via a maternal line

Unaffected male sibling of affected male

Availability for clinical examination

1.5.4.1 Case series study - clinical component:

This part of the study will involve re-examination of the patients and their relevant family members. Pedigrees will also be obtained during this process. A data capture sheet will be designed to help facilitate information gathering. Medical records will be reviewed.

(appendices 1 and 2)

1.5.4.2 Case series study – molecular component:

This part of the study will be carried out in the University of Cape Town, Division of Human Genetics laboratory.

a) DNA extraction

DNA will be extracted from participants' peripheral blood or saliva samples. DNA from an unaffected male sibling will also be extracted from saliva if he and his mother agree to participation. DNA will be extracted from blood using a salting out method, according to the laboratory operating protocol. DNA from saliva will be extracted using DNA genotek extraction kit.

b) Primer design and PCR

Each of the eight exons of *GPC3* will be amplified during a Polymerase Chain reaction (PCR) using primer pairs. Exons 1-8 will be amplified using forward and reverse primers that have previously been described by Sakazume et al. Exon 3 will be amplified with an additional internal primer pair due to the large size of this exon¹⁰. These primers were designed to overlap the intron exon boundary. The primers were checked for amplification specificity and stability.

c) Mutation identification

The PCR amplification products will initially be run on an agarose gel. If a whole exon deletion is present there will be no band visible on the agarose gel. A control sample will also be used as this will confirm whether the PCR amplification process was successful. The control samples that will be used will be that of an unrelated, unaffected male, and proband S's unaffected brother (if consent is obtained). If a whole exon deletion is found to be present, a literature search using PUBMED will be undertaken to confirm that this deletion has previously been reported and is thus disease-causing. If this is found to be disease-causing further analysis of at risk family members will be undertaken, starting with the relevant mother.

If no obvious deletion is identified the PCR products will be sequenced using a sequencing kit and then analysed using an ABI 3130 sequence analyser. If a mutation is identified it will be investigated by comparison with an unaffected male reference sample. It will also be referenced against literature reports on known mutations in SGBS and compared to bioinformatics databases to assess putative implications. If it is found to be disease-causing further investigation of at risk family members will be undertaken.

If no obvious mutation is found during sequencing the diagnosis can neither be confirmed nor refuted.

(appendices 3 and 4 illustrate flow diagrams explaining the methodology)

1.5.5 Analysis

A careful description of relevant clinical features will be presented and compared with other family members and previously reported patients.

Molecular findings will be analysed by using software programs. The results will then be compared to the current reference sequence of *GPC3* on the NCBI website.

1.6 ETHICS

Written informed consent will be obtained from all participants. The home languages of the participants are English and Afrikaans and informed consent will be available in both these languages. In the case of minors, consent will be obtained from their parents and where possible assent will be obtained from children. An information sheet will also be given to all participants to take home. (appendices 5, 6 and 7)

All information will be stored in password protected computers. Written information will be stored in a locked office.

All personal identifiers will be changed if the data is published. Photographs will only be used with the eyes hidden and with informed consent.

There is no significant risk of physical harm during this study. No other genes or genetic information, other than that related to SGBS, will be analysed.

Participation in the study will be on a voluntary basis. Should any participants wish to discontinue their involvement in the study they could do so at any time.

Contact details of the researcher and the supervisor will be supplied on the information sheet.

The results of the analysis and the implications thereof will be conveyed to the families, either personally or telephonically if a face to face consultation is not possible.

All efforts will be made to inform participants of results.

Ethics approval from the Research Ethics Committee of the University of Cape Town will be sought.

1.7 LOGISTICS

Table 1.1: Planned time frame of project

Protocol writing	December 2011
Ethics submission	January 2012
Literature review	December 2011 – January 2012
Clinical information gathering	February 2012 – March 2012
Molecular laboratory work	March 2012 – May 2012
Analysis of data	May 2012 – June 2012
Write up	July 2012 – September 2012
Submission	October 2012

1.8 BUDGET

National Research Foundation funds are available for the project. (appendix 8 for the funding application)

Table 1.2: Proposed budget for research project

MOLECULAR INVESTIGATIONS:	
Extraction DNA	R 500.00
Primers for amplifying exons	R 5000.00
PCR	R 500.00
Sequencing	R 10 000.00
MISCELLANEOUS:	
Printing and binding	R 600.00
Miscellaneous	R 400.00
PROPOSED BUDGET TOTAL:	R 17 000.00

1.9 REFERENCES

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2 LITERATURE REVIEW

2.1 OBJECTIVES

The objective of the study was to investigate two boys with Simpson-Golabi-Behmel syndrome (SGBS)[OMIM #312870]. The clinical features in these boys were documented and investigations were undertaken to identify the molecular mutation causing the disorder in the two families.

The main objectives of this literature review are:

- To find reports that describe the clinical features of SGBS.
- To ascertain whether there are any population specific differences in the clinical presentation of SGBS
- To investigate the genetic basis and inheritance of SGBS
- To review the methods through which a molecular diagnosis can be made in this condition.

2.2 LITERATURE SEARCH STRATEGY

An internet search was undertaken using the PUBMED database. The search was broadly divided into two parts. The first part related to the clinical presentation, and the second part was focused on the genetic transmission and molecular basis of the disorder.

In the clinical part of the internet search the following phrases were used:

“Simpson Golabi Behmel AND clinical”

“Simpson Golabi Behmel AND diagnostic features”

“Simpson Golabi Behmel AND diagnostic criteria”

The first search produced 40 reports, and the second only 12. No reports were found for the third search criteria.

The literature was included or rejected according to the following criteria.

Inclusion criteria:

- Studies describing the clinical features of affected persons.
- Recent review articles
- Articles written in English

The first study describing SGBS was published in 1975. For this reason there were no specific inclusion or exclusion criteria relating to timing of publishing.

Exclusion criteria:

- Studies describing the clinical features of Simpson-Golabi-Behmel syndrome type 2. This condition is sometimes referred to as the lethal variant of SGBS. The causative mutation in this syndrome maps to a different genetic locus (Xp22)^{1,2} and is presumed to be a distinct disorder³.

In the search addressing the molecular genetic basis of SGBS the following phrases were used:

“Simpson Golabi Behmel AND molecular”

“Simpson Golabi Behmel AND *GPC3*”

“Simpson Golabi Behmel AND *GPC4*”

The first search produced 53 articles, and the second 73. The search for “Simpson Golabi Behmel AND *GPC4*” yielded only 11 articles.

Inclusion criteria:

- All data published from 1996 and onward. The gene responsible for SGBS was identified in this year.
- Only studies describing *GPC3* and *GPC4* in relation to SGBS were included.

Exclusion criteria:

- Studies relating solely to animal subjects
- Studies describing only the function of *GPC3* and *GPC4*.
- Studies describing the molecular basis of Simpson-Golabi-Behmel syndrome type 2.(see above for rationale)

Neither clinical nor molecular studies describing South African patients with SGBS could be found on PUBMED.

2.3 LITERATURE REVIEW

2.3.1 Introduction

Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked congenital abnormality overgrowth syndrome. The condition was described for the first time in 1975 by Simpson et al who described their patients as having “bulldoglike” facies, with a wide nasal bridge and anteverted nares⁴. Behmel et al commented that these boys had a wide mouth and protruding tongue, with a midline depression in the lower lip. The affected individuals had a protruding maxilla and broad hands. X-linked inheritance was suggested as the affected individuals were boys, born to unaffected mothers⁵.

In January 1984 Golabi and Rosen published an article in which they described four males with multiple congenital abnormalities and mental retardation. These individuals had prenatal macrosomia and shared similar facial features such as a short broad upturned nose, a large mouth with a groove in the lower lip and a cleft palate. They also had skeletal abnormalities and various renal, cardiac and gastrointestinal malformations. The authors postulated that it was a previously undescribed syndrome⁶.

A short while after this article was published, Behmel et al⁵ reported on 13 males with increased birth length and weight, together with short hands and feet. The typical features these authors described were coarse facies, short upturned nose, wide open mouth with a large tongue and midline depression of the lip, and a prominent jaw. Normal intelligence was reported in all their living patients. In their discussion it was mentioned that although their cases shared similar facies to those previously described by Simpson and by Golabi and Rosen, there were differences in terms of certain features and intelligence⁵.

In 1988 Neri et al suggested that all three of the above papers described the same syndrome and coined the term “Simpson-Golabi-Behmel syndrome”⁷. The exact prevalence of SGBS is unknown, but it is universally accepted that this condition is under-diagnosed³. This may be because SGBS is a relatively newly described syndrome together with a lack of commercially available molecular testing. Furthermore, it is primarily a clinical diagnosis and there is considerable phenotypic overlap with other overgrowth syndromes⁸. Nevertheless, at least 130 cases have been documented in the literature⁹.

The recognition and diagnosis of patients with SGBS is important because:

- Affected boys have an increased risk of embryonal tumour development and need surveillance^{5,10}.
- People with SGBS are at risk of cardiac, genito-urinary and gastrointestinal complications. Skeletal and developmental abnormalities also occur¹¹.
- Identifying the disorder can help to determine the recurrence risks in siblings and in the family.

2.3.2 Clinical features

The diagnosis of SGBS is based on clinical manifestations. These features can be variable and difficult to identify but the diagnosis warrants consideration in the presence of a distinctive facies, multiple abnormalities and overgrowth¹².

In only one of the articles reviewed have diagnostic criteria been used to assist in defining the cohort that the authors were studying. In this report the males with overgrowth and at least two additional traits were considered as having SGBS. The additional features were:

- Characteristic facial appearance
- Hand anomalies
- Skeletal abnormalities
- Accessory nipples
- X-linked inheritance pattern¹³

Antenatal findings

Prenatal ultrasound investigation has facilitated the ability to make a prenatal diagnosis of SGBS. This condition is one of a few overgrowth disorders that present antenatally with macrosomia. There are, however, other features that may help in distinguishing SGBS from the other overgrowth disorders. Antenatal findings that have been reported in association with SGBS are increased nuchal translucency, congenital diaphragmatic hernia, visceromegaly, postaxial polydactyly and a single umbilical artery. Renal abnormalities including enlarged kidneys, hydronephrosis and hydroureters have also previously been reported^{14,15}. When macrosomia and any of these abnormalities are found together with polyhydramnios and an elevated maternal serum alpha feto protein (msAFP), SGBS warrants consideration¹⁵.

Growth parameters

Macrosomia is a common feature of SGBS. This parameter is defined as a birth weight and height greater than the 97th centile for gestational age. Pre- and postnatal overgrowth occur in SGBS⁹. The final adult height is often more than 2 m, depending on parental height⁷.

Facial features

In the papers describing the first boys with SGBS they were said to have a broad and stocky appearance. Their facial appearance was described as coarse, with hypertelorism and a broad nasal bridge⁶. The jaw and maxilla were prominent, and the mouth wide and open with a groove in the lower lip⁶. The nose was short and upturned. Submucous palatal clefts and bifid uvula were mentioned⁶.

In the articles following these original descriptions, the typical SGBS facies is consistently described as coarse. People with SGBS have hypertelorism with a heavy brow, downslanting palpebral fissures and epicanthic folds^{7,8,9,12,14,16,17,18}.

The nose is short, and upturned^{7,9,14,18}. Affected boys often have macrostomia with a groove in either the tongue or the lower lip^{7,8,9,11,12,18,19}. In the original descriptions macroglossia was not mentioned, but several later observations include macroglossia as a feature of SGBS^{7,8,9,12,13,14,16,17,18,19,20}. Dental malocclusion can occur due to the macroglossia^{17,18}. The prognathism which was originally described is also mentioned in later reports.

Ear abnormalities described vary and include low set ears, thick auricles and creased lobes^{9,14,17,20}.

Cleft lip and palate are present in about 25% of affected persons²⁰. Other palatal abnormalities were mentioned in some studies, notably a high arched^{5,10,12,16,20,21} or narrow palate⁹.

Other associated abnormalities

Multiple other abnormalities have been described in association with SGBS. Short, broad hands with polydactyly, cardiac defects, hernias and variable skeletal abnormalities have all been documented. Genito-urinary and gastrointestinal anomalies occur. Intellectual capacity is variable^{5,6}.

The malformations that have been described in the case reports which have been reviewed are summarised in table 2.1.

Table 2.1: Clinical features described in literature.

	Gurrieri et al 2011 ¹⁰	Ratbi et al 2010 ¹⁸	Lindsay et al 1997 ¹¹	Griffith et al 2009 ¹⁷	Rodriguez-Criado et al 2005 ⁹	Li et al 2001 ¹³	Mariani et al 2003 ²⁰	Okamoto et al 1999 ¹²	Young et al 2006 ¹⁶	Yano et al 2011 ¹⁴
Supernumerary nipples	Yes	Yes		3/3	4/6	3/6	Yes			0/2
Cardiac	Patent foramen ovale	Normal	8/18 with abnormality	3/3 Normal	1/6 pulmonary hypertension with right ventricular hypertrophy	1/7 cardiac abnormality	Tricuspid valve anomaly			1/2 ventriculo-septal defect
Renal	Normal	Normal	7/18 with abnormality	3/3 Normal	2/6 abnormalities ⁰	4/7 abnormalities	Nephromegaly			2/2 Nephromegaly and hydronephrosis
Digital	Short fingers, mild webbing	Postaxial polydactyly		3/3 Broad fingertips	1/6 short hands	5/7 hand anomalies	Prominent and splayed fingertips with bilateral simian creases		Broad flat hands	2/2 Large
Feet	Normal				3/6 abnormal*		Pes planus		Lateral deviation toes	
Skeletal abnormalities			9/18 abnormalities		1/6 pectus carinatum	3/7	Supernumerary rib and pectus excavatum			2/2 pectus excavatum and rib/vertebral abnormalities
CNS abnormalities		None	2/18 patients with abnormality		1/6 corpus callosum agenesis		Large adeno-hypophysis		Hydrocephalus	
Genitourinary abnormalities		Undescended testes		3/3 undescended testes 1/3 penoscrotal hypospadias		1/7 inguinal hernia	Undescended testes	Inguinal hernia	Inguinal hernia	1/2 hydrocele
Gastrointestinal abnormalities		Normal			1/6 hepatosplenomegaly	3/6 hernias √	Hepatosplenomegaly	Congenital diaphragmatic hernia	Umbilical hernia	
Development	Mild delay		17/18 patients delayed	1/3 motor delay	2/6 motor delays	2/6 developmental delay	Normal		Severe developmental delay	2/2 speech and motor delay

Key: *pes planus, broad halluces, pes varus ⁰ Ureterohydronephrosis, large kidney √diaphragmatic and umbilical

Syndromic features of SGBS consistently reported in the literature are outlined below.

Skeletal anomalies

The hands can show brachy-, campto- or clinodactyly. Varying degrees of postaxial poly- and syndactyly of the hands and feet can be present^{7,8}. The hands and feet are often short and broad^{7,16}.

Vertebral abnormalities and scoliosis of the spine may occur as does pectus excavatum⁸.

Gastro-intestinal anomalies

Organomegaly and various intestinal malformations such as malrotation, pyloric rings and Meckel's diverticulum have been described. Diaphragmatic and umbilical hernias as well as omphaloceles may be present^{7,8,10,17}.

Renal anomalies

Enlarged, hydronephrotic, cystic or dysplastic kidneys can occur^{7,8,13}. Renal abnormalities may be detected antenatally. This finding in the presence of overgrowth can lead to the inclusion of SGBS in the differential diagnosis.

Genito-urinary anomalies

Minor genital abnormalities are common. These include cryptorchidism and inguinal hernias. Hypospadias and ambiguous genitalia are occasionally associated with SGBS¹⁷.

Cardiac anomalies

Congenital heart defects in SGBS have been well described in a number of studies. A cardiac defect can be found in up to 36% of patients with SGBS¹⁹. The majority are structural cardiac malformations, but cardiomyopathies and isolated ECG abnormalities are also described. There is uncertainty as to whether or not the ECG abnormalities reflect a true association with SGBS or just selection bias¹⁹. The structural cardiac anomalies are mostly atrioseptal and ventriculoseptal defects or simple valve defects¹⁹.

Neurological abnormalities

The neurological features in SGBS vary widely but hypotonia is the most consistent neurological finding in SGBS⁶. Functional and cognitive outcomes can be normal in some boys, whereas developmental delay occurs in others. Speech delay is present in 50%, and motor delay in 34% of affected individuals. Severe intellectual disability is rare²⁰.

Seizures and EEG abnormalities have been described^{7,8}. Structural central nervous system abnormalities include Dandy-Walker malformations and agenesis of the corpus callosum^{8,9}.

Other manifestations

The fingernails can be broad and dysplastic¹⁹.

2.3.3 Neoplasia

SGBS belongs to the group of overgrowth syndromes in which a common factor is the risk of development of a tumour¹⁰. In particular in SGBS there is a clearly defined increased risk in developing embryonic cancers^{7,18} especially of the kidney and liver⁹. Wilms tumour, neuroblastoma, hepatocellular carcinoma, hepatoblastoma and testicular gonadoblastoma are the tumours that have been documented. Although it is widely acknowledged in the literature that risk for malignancy is sufficiently increased to warrant tumour surveillance^{8,13}, only a few studies quote risk figures. These range from 5% to 15%^{9,14,20}.

2.3.4 Population specific phenotype

Most reports in the literature do not state the population or ethnic group of the affected individuals. The studies that did mention the ethnic background of their patients revealed considerable diversity, ranging from the original report by Golabi concerning a Puerto-Rican boy, to reports from Europe (Germany, Italy, Holland), Australia, Angola, the Caribbean, Jordan and Japan^{6,9,10,11,12,14}. The phenotype in these studies was comparable leading to the reasonable assumption that there is no significant difference in clinical presentation between different population groups.

During the literature search no reports of SGBS in the South African population could be found.

2.3.5 Genetic basis and inheritance

Introduction and inheritance

In the original reports describing SGBS it was already recognised that there was an X-linked recessive mode of inheritance. Affected individuals were male, and females showed no or only mild features of SGBS^{5,6}. This inheritance pattern is often useful in distinguishing SGBS from the other overgrowth syndromes.

The main indicator of recurrence risk would be the carrier status of the mother of the affected boy. If she is a carrier of the determinant gene, the recurrence risk would be 50% for any further male offspring. If she is not a carrier the recurrence risk would be very low. The authors Hughes-Benzie et al, in 1996, suggested that it may be that new mutations are responsible for the majority of males with SGBS²¹. In contrast to this, these authors then go on to recommend that for counselling purposes it may be empirically assumed that one in three affected boys are due to a new mutation, meaning that two in three mothers will be carriers of the mutation. The authors felt that more research is needed to confirm this²¹. A molecular diagnosis greatly improves the accuracy of recurrence risk.

Genetic background

Two genes have been implicated in SGBS. In 1994 the region was mapped to Xq25-27²². In 1996 the first gene involved in SGBS - glypican 3 (*GPC3*) - was identified by investigating the breakpoints on the X-chromosomes of two affected females with X-autosome translocations²³.

Adjacent to *GPC3* is glypican 4 (*GPC4*); previous reports have shown that deletions in *GPC3* can extend to *GPC4*². Recently, an isolated *GPC4* duplication has been implicated as a second locus for SGBS²⁴.

GPC3

The gene *GPC3* at Xq26 contains eight exons, and is 500 kilobases long. It belongs to the glypicans family; which are heparan sulfate proteoglycans that attach to the cell surface by glycosylphosphatidylinositol anchors¹⁶ where they are involved in the signalling of morphogens and heparan-binding growth factors. Glypicans are expressed in mesodermal tissues, such as the kidneys, liver and lungs⁹. *GPC3* can also interact with insulin-like

growth factor 2 (IGF2), which is involved in another overgrowth syndrome, Beckwith-Wiedemann Syndrome.

The mutation spectrum within *GPC3* causing SGBS is broad, ranging from deletions to point mutations. The original report describing *GPC3* mutations was that of three different deletions⁷, and in further studies additional deletions were identified. Deletions can involve one or more exons, or may be a microdeletion within an exon^{2,11}. In 50% of persons with a deletion, this involves exon eight and extends to the centromere². Large deletions are found in up to 20% of persons with SGBS²⁵.

With advances in molecular technology, it has been possible to further investigate *GPC3*. Direct sequencing has identified numerous different point mutations, while intronic mutations affecting the splice site have also been reported⁹. Point mutations are most frequently found in exon 3, which is the largest exon encompassing 40% of the gene. There is no evidence for hot spots for mutations².

The deletions and point mutations which have been documented to date all introduce premature stop codons resulting in an unstable protein². This truncated protein has reduced adherence to the cell surface. It is suggested that deletions and point mutations are equally common in SGBS⁹ and that the majority are unique.

Not all individuals with SGBS have been found to have a mutation in *GPC3*. The question therefore remains as to what the cause for SGBS in these affected persons is. The possibilities include other mechanisms of gene silencing, the involvement of promotor areas, or that there are more genes involved¹³. Different studies have reported mutation detection rates varying from 37%²⁰ to 70%^{2,19}.

GPC4

The gene *GPC4* is located next to the 3' end of *GPC3* on the X-chromosome. *GPC4* is also a part of the heparan sulfate proteoglycans family and is also bound to the cell surface by glycosylphosphatidylinositol.

Deletions encompassing both *GPC3* and *GPC4* have previously been reported in SGBS. No deletions involving *GPC4* alone have been found in males with SGBS, and neither have point mutations in the *GPC4* gene².

A recent study revealed a duplication of exon 1 to 9 of *GPC4* in a male with SGBS²⁴. In this family it was confirmed as disease-causing when it was shown to segregate with other clinically affected males. This is the first time that an isolated *GPC4* mutation has been identified in a family with SGBS. The exact pathogenic mechanism of *GPC4* duplications requires further investigation²⁴.

In previous investigations *GPC4* polymorphisms had been identified, but found not to cause loss of function and therefore assumed not to be disease-causing in SGBS².

Genotype – phenotype correlation

SGBS is likely the result of a loss of function mechanisms^{2,20,21}. Thus far there has been no correlation between the genotype and the phenotype. The size of the deletion is also not related to the severity of the clinical features¹⁷. The phenotype is highly variable even within families where affected males share the same molecular mutation².

2.3.6 Testing strategy and methods

The information in this review seems to indicate that it is necessary to investigate *GPC3* for both deletions and point mutations. A testing strategy has been suggested in GeneReviews³, starting with sequence analysis of *GPC3*, followed by deletion/duplication analysis of *GPC3*. If no mutation is found, deletion and duplication testing of *GPC4* should be undertaken.

Investigations undertaken just after the identification of the gene involved in SGBS relied on polymerase chain reaction (PCR) amplification of exons 1 to 8, and Southern blotting to identify deletions²¹. Both DNA extracted from lymphocytes and fibroblasts have been used. The primers commonly employed in earlier studies are those defined by Pilia et al in 1996²³, and Huber et al in 1997²⁶, although lately most investigators have added either intronic primers, or redefined primers^{2,11,12}. Newly designed primers covering all eight exons, and including intronic-exonic borders are now in use. These primers also include an internal primer set in exon 3 to better cover this large exon²⁵. After amplification the products are evaluated by electrophoresis on various gels². Thereafter, sequencing of the amplified products in forward and reverse directions is conducted and these results analysed using different software programs¹⁰. Mutations are confirmed by repeating PCR amplification and sequencing²

2.4 CONCLUSION

This literature review confirms that there have been many reports documenting the clinical phenotype of SGBS and that many of the manifestations are relatively nonspecific and can be found in various other syndromes. The combination of macrosomia, characteristic facial features and multiple congenital abnormalities is suggestive of SGBS. A comprehensive review of the literature concerning the clinical phenotype of SGBS supported the diagnosis of SGBS in the boys in the existing study.

No data from South Africa regarding SGBS could be identified in this literature search. Although no apparent phenotypic differences between population groups are described, the importance of documenting the phenotype of South African patients would be to raise awareness of the diagnosis and to improve clinical diagnostic skills in this country.

The genetic basis of SGBS has only been elucidated in the past two decades and determinant mutations are not identifiable in all persons with SGBS. The deletions and point mutations identified to date have all been unique and there is still a need for further investigation into the mutation spectrum. This literature review confirms that there is a good chance of finding a mutation in *GPC3* in persons with SGBS. There has been only one reported case of a *GPC4* duplication causing SGBS and currently no other genes are known to be implicated in the condition.

The practical implication of this investigation will be that if a mutation in *GPC3* is identified in the two probands, the clinical diagnosis of SGBS will be confirmed. It would then be possible to proceed to offer testing to at risk family members to establish their individual risk. Establishing the methodology for *GPC3* sequencing would facilitate future establishment of diagnostic testing facilities for SGBS in South Africa.

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3 ABSTRACT

3.1 Study rationale

Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked overgrowth syndrome. It is characterised by macrosomia, distinctive facial features, and multiple congenital abnormalities.

Two genes have been found to be associated with SGBS. They are glypican 3 (*GPC3*) and glypican 4 (*GPC4*). Mutations in *GPC3* are detected in 37-70% of affected males.

The aim of this research was to describe the phenotype of two unrelated boys and to attempt to make a molecular diagnosis in their families by investigating *GPC3*.

3.2 Methods

This study is a case series with a clinical and molecular component. Two male probands were identified, proband B and S. Their clinical records were reviewed to obtain relevant history and their physical manifestations were documented.

DNA was extracted from proband B and S as well as their mothers. All eight exons of *GPC3* were amplified by polymerase chain reaction (PCR). The products were first analysed for large gene deletions and thereafter sequencing analysis was undertaken to identify point mutations.

3.3 Results

The clinical phenotype of proband B and S was documented and found to be consistent with that reported in the literature.

DNA analysis of proband B revealed a mutation in exon 4 of *GPC3*. This mutation consisted of a deletion of four nucleotides, TAGA, at nucleotide position 1071, and an insertion of three nucleotides, CTT. This mutation can be labelled as p.358Arg-PheFSX373 (NM_004484.3).

No deletion or mutation in *GPC3* was identified in proband S.

3.4 Discussion

The boys who were included in this investigation exhibited many of the more common features seen in SGBS.

Both probands B and S had characteristic facial features and showed varying degrees of pre- and postnatal macrosomia.

These two boys also exhibited skeletal and cardiovascular abnormalities that have been described in SGBS. Proband B had hypospadias which can occur in SGBS. Proband S had visceromegaly which can also be a feature of SGBS. Proband B developed a Wilms tumour, which is a cancer that is associated with SGBS in the literature.

The main clinical manifestations which prompted a diagnosis of SGBS in the two boys were macrosomia, coarse facial features, macroglossia and a grooved tongue.

The molecular analysis of proband B's DNA revealed a frameshift mutation resulting in a premature stop codon. This stop codon occurs in exon 4 meaning that the majority of the protein is not translated. This is a previously undescribed mutation.

3.5 Conclusion

This report represents the first published description of South African patients with SGBS.

The phenotype of the boys included in this research is similar to that previously reported in the literature. On this basis clinicians in South Africa can be guided by the literature in the diagnosis of SGBS.

The importance of regular tumour surveillance is reinforced in this research by virtue of the Wilms tumour that proband B developed.

The mutation found in proband B represents a novel, and likely disease-causing mutation. The literature supports this statement as the functional effect of this mutation, a premature stop codon, has been described numerous times.

4 MANUSCRIPT

4.1 INTRODUCTION

Phenotype

Simpson-Golabi-Behmel syndrome (SGBS)[OMIM #312870] is an X-linked overgrowth syndrome. It is characterised by macrosomia, distinctive facial features, and multiple congenital abnormalities¹. The condition was originally reported by Simpson et al² and similar features were described in males seen by Golabi and Rosen³, and by Behmel et al⁴. In 1988 Neri coined the term “Simpson-Golabi-Behmel syndrome” after recognising that the previous authors had been describing the same syndrome⁵.

It is widely accepted that SGBS is under-diagnosed, probably because it is a relatively newly described syndrome and there is clinical overlap with the other overgrowth syndromes⁶.

The SGBS is typically characterised by pre- and postnatal overgrowth, although not all affected persons have postnatal overgrowth⁷. In a newborn with macrosomia this diagnosis warrants consideration if there are the typical facies or renal or skeletal abnormalities⁸. In the majority of cases prenatal overgrowth, coarse facies, supernumerary nipples, and hand anomalies are present⁷.

The typical facial features include a coarse appearance, hypertelorism, and downslanting palpebral fissures. The nose is often upturned, and macrostomia and macroglossia are usual. Grooves in the tongue and lower lip are also common findings.

The hands are broad and short, and poly- or syndactyly can occur. Other skeletal manifestations include scoliosis, vertebral abnormalities, and pectus deformities.

Genito-urinary abnormalities in SGBS are usually mild and include cryptorchidism and inguinal hernias, but more serious abnormalities such as penoscrotal hypospadias and ambiguous genitalia have been described⁹.

Abdominal anomalies which can occur include intestinal malformations and umbilical or diaphragmatic hernias.

Renal involvement is not uncommon. The kidneys are hydronephrotic in 15% of affected individuals and may be polycystic or enlarged. Nephromegaly or renal dysplasia occurs in 31% of persons with SGBS⁷.

Cardiac abnormalities are present in up to 36% of patients with SGBS. The majority of these are structural defects. Cardiomyopathies and electrocardiogram (ECG) abnormalities also occur¹⁰.

Structural neurological abnormalities have been described, such as Dandy Walker malformations and corpus callosum anomalies. Hypotonia is frequent⁶. The developmental outcome is variable, ranging from normality or mild learning difficulties, to infrequently severe intellectual dysfunction. Speech delay has been documented in 50% of boys with SGBS, and motor delays in 34%⁷.

The SGBS is associated with an increased risk of developing embryonal tumours. Carcinoma has been described in 10 affected children, and in every instance the tumour was intra-abdominal¹¹. The tumour spectrum included four Wilms tumours, three hepatoblastomas, one adrenal neuroblastoma, one gonadoblastoma and one hepatocellular carcinoma. An elevated risk of developing a tumour has been well documented and it is generally accepted that the risk is high enough to warrant tumour surveillance protocols to be put in place. Lapuzina et al (2005) have quoted this risk to be up to 10%¹¹.

Genotype

Two genes have been found to be associated with SGBS. They are glypican 3 (*GPC3*) and glypican 4 (*GPC4*). *GPC3* was the first gene identified. This discovery was made while investigating affected females with X-autosome translocations¹². The gene is located at Xq26, and encodes a heparan sulfate proteoglycan which is involved with growth factor signalling and morphogenesis. *GPC3* is expressed in tissues of the mesoderm, notably the kidneys, liver and lungs¹³. *GPC3* interacts with insulin-like growth factor type 2 (IGF2) and can alter its signalling activities¹⁴. It has been postulated that *GPC3* and IGF2 form a complex, and that *GPC3* could be involved in downregulating IGF2 via the IGF2 receptor. This has however not been confirmed in subsequent studies⁷. The mutation spectrum is broad, but most cases of SGBS are due to either deletions or point mutations¹⁴. There are no known hot spots, and mutations can occur throughout the gene¹⁵. All of these mutations have a loss of function effect, and there is no genotype-phenotype correlation⁷. Mutations have been reported to be detectable in 37-70% of patients¹.

GPC4 is adjacent to the 3' end of *GPC3*. Previously deletions involving *GPC3* have been reported to extend to *GPC4* as well. *GPC4* mutations alone have not been associated with SGBS until recently when a duplication of exons one to nine of *GPC4* was found to be disease-causing in a family with SGBS¹⁶.

SGBS is inherited in an X-linked recessive manner. Carrier females are usually unaffected but may exhibit some minor clinical features, such as tall stature¹ with a short broad nose and a wide mouth⁴.

Research aims

The aim of this research was to describe the phenotype of two unrelated boys and to attempt to make a molecular diagnosis in their families by investigating *GPC3*. It is hoped that this project will improve clinical diagnostic ability by delineating the phenotype encountered in our South African patients. Furthermore, it will also be investigated whether there are any significant differences between published literature and the affected boys investigated. Identifying the disease-causing mutation would not only confirm a clinical diagnosis, but is also necessary to give an accurate recurrence risk to the parents. If a disease-causing mutation is found it would be possible to make diagnostic testing available to other at risk family members. Currently, molecular analysis of *GPC3* is not readily available to patients in the public health arena in South Africa.

4.2 METHODS

The type of study undertaken was a case-series with a clinical and molecular component. For the sake of expediency the two boys who participated in the study were named proband B and proband S, belonging to family B and S respectively. B and S represents the first letter of each individual proband's first name.

4.2.1 Patient selection

Proband B was ascertained after referral to the Division of Human Genetics of the University of Cape Town. Proband S had been attending the Genetic Clinic at Red Cross War Memorial Children's Hospital for several years. In both these boys a clinical diagnosis of SGBS was made in the genetic clinic after careful review of the clinical features and the literature. These individuals were seen between January 2011 and May 2012. They were the only patients in whom a diagnosis of SGBS was considered likely during this period.

The inclusion criteria were

- Boys clinically affected with SGBS
- Availability for clinical examination
- Mothers of affected boys who were available for examination

4.2.2 Ethical approval

Ethical approval was sought and granted from the Human Research Ethics committee, Faculty of Health Sciences of the University of Cape Town. (appendix 9) The eldest of the two probands was five years of age and the other was aged four years. Both mothers gave written consent for themselves, and their children to participate in the research. Assent from an affected child is only required after the arbitrary age of seven years. Both probands are under this age and were not considered emotionally mature enough to be able to understand the pertinent information.

4.2.3 Phenotypic documentation

Hospital records were reviewed to obtain a family history, clinical information, and results of special investigations. Clinical consultations were arranged with both mothers and their sons. A detailed clinical examination was performed on both mothers and their sons by the investigator. A clinical information sheet was designed to facilitate directed questions regarding demographic data, antenatal, birthing, postnatal and developmental history. The documentation of phenotypic features was aided by a tick sheet designed to incorporate the typical features of SGBS as described in the literature. The sheet included a space to elaborate if a clinical feature did not appear on the tick sheet. Provision was also made for recording investigations which had been undertaken for tumour surveillance. (appendices 1 and 2)

4.2.4 Molecular analysis

The molecular analysis was undertaken by the investigator in the Ramesar Laboratory, Division of Human Genetics, University of Cape Town (UCT).

A flow diagram was designed to aid in the molecular diagnostic investigations. The testing strategy used is similar to those previously described in the literature¹⁷. This approach was also the most cost-effective way to proceed with testing. The flow diagram can also assist in determining the course of action, once a mutation has been identified (appendices 3 and 4).

4.2.4.1 GPC3 polymerase chain reaction (PCR) assay design and optimisation

The primers selected for the amplification of *GPC3* coding regions were those reported on in 2007 by Sakazume et al (Table 4.1)¹⁷. These primer pairs were investigated for their suitability by evaluating their amplification specificity and also their tendency for secondary structure formation. Initially, the primer sequences were submitted to Primer-BLAST¹⁸ to determine that they were indeed specific to the area of interest. Secondly, they were analysed using OligoAnalyzer 3.1¹⁹ to assess their stability. Exon 3 was analysed with two overlapping sets of primers due to the large size of the coding region. These primer sets were termed 3A and 3B.

Table 4.1: Primers used for the amplification of *GPC3* coding regions (Sakazume et al [2007])¹⁷

Gene region	Primer sequence (5' – 3')	Size of product (bp)
<i>GPC3</i> exon1 F	CTCAGGGTACAGCCACCAC	399
<i>GPC3</i> exon1 R	CAGGTAGCTGCGAGGAAACT	
<i>GPC3</i> exon2 F	GCCCAAATAATGATGCCACT	277
<i>GPC3</i> exon2 R	GGTGTGGGTGTGTGAGAGAG	
<i>GPC3</i> exon3a F	TACCTGCTACTGGCCACCTC	493
<i>GPC3</i> exon3a R	GAGCAAGACGTGACCTGAAA	
<i>GPC3</i> exon3b F	CGGCCACAGTCCTTACTGA	501
<i>GPC3</i> exon3b R	TTTTCACACTGGATTTTCATGC	
<i>GPC3</i> exon4 F	TTTCACTCTAGTGGTTTTTGACCTT	302
<i>GPC3</i> exon4 R	TGGGGGAAGAAATTGAAGTG	
<i>GPC3</i> exon5 F	TTTCTGGTGCAATTAATGGAGA	267
<i>GPC3</i> exon5 R	TTGCCTCTTATGCACAGATGTT	
<i>GPC3</i> exon6 F	CTCTCTCTCTCCCCCTCCTC	255
<i>GPC3</i> exon6 R	GCTTTTCCTTTGTTTGGGACT	
<i>GPC3</i> exon7 F	TTGTGTGTTGCAGGGAATGT	327
<i>GPC3</i> exon7 R	TGCAGACCCACCTGAGAAAT	
<i>GPC3</i> exon8 F	GCTCGAGCTGTGCATAGTGT	306
<i>GPC3</i> exon8 R	CCCTTTATCGAGGAAGACCAC	

Key: bp – base pair(s)

DNA was extracted by the Division Human Genetics, UCT for proband S and his mother, whilst the National Health Laboratory Services (NHLS), Groote Schuur Hospital (GSH), extracted DNA for proband B and his mother. The extraction method employed by the NHLS involved the QIAamp DNA blood mini kit (Whitehead scientific, Brackenfell, South Africa). In the Division Human Genetics, DNA was extracted from peripheral blood lymphocytes using the salting-out method described by Miller et al²⁰.

The DNA was then assessed for its quantity and quality by spectrophotometry (NanoDrop® ND-1000, Thermo Fisher Scientific, Wilmington, Delaware, USA) and electrophoresis on a 1.5% (w/v) agarose (Lonza, Basel, Switzerland) gel.

The PCR assay was optimised using DNA obtained from a healthy research participant. During this process the optimal temperature was investigated on a Bio-Rad T100™ gradient thermal cycler (Bio-Rad, Hercules, California, USA), and titrations were performed for Magnesium Chloride (MgCl₂) and pH to enhance primer annealing and the amplification yield of products (Table 4.2).

Table 4.2: Assay optimisation

GPC3 region	Temperature	MgCl₂ and pH titration
Exon 1	62.7°C	1.5mM MgCl ₂ and pH 8.5
Exon 2	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 3	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 3A	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 3B	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 4	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 5	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 6	59°C	1.5Mm MgCl ₂ and pH8.6 (Buffer no 6 of PCR optimisation kit, Roche)
Exon 7	59°C	1.5mM MgCl ₂ and pH 8.5
Exon 8	59°C	1.5mM MgCl ₂ and pH 8.5

4.2.4.2 Amplification of GPC3

Standard conditions for PCR amplification:

The DNA of both participants was amplified in a final PCR volume of 25 μ l. For all the *GPC3* exon regions, except for exon 6, this reaction consisted of 100ng of genomic DNA, 1x GoTaq® (Promega, Madison, WI, USA) reaction buffer, 200 μ M of deoxyribonucleotide triphosphates (dNTP) (Bioline, London, UK), 0.4 μ M of each primer (sense and antisense) and 0.5 units of GoTaq® DNA Polymerase (Promega). For *GPC3* exon 6 amplification, the PCR Optimization Kit Buffer number 6 (Roche, Basel, Switzerland) was used.

The PCR reactions were performed on the T100™ thermal cycler (Bio-Rad Laboratories). It consisted of an initial DNA denaturation step (95°C for 5 minutes), followed by amplification over 35 cycles. Each amplification cycle consisted of 30 seconds of denaturation at 95°C, 30 seconds of primer-template annealing (at the optimal temperatures in table 4.2), 40 seconds of product extension at 72°C. Finally, a cycle of 7 minutes at 72°C allowed for the extended synthesis of incomplete DNA fragments.

The yield and specificity of the aforementioned reaction were investigated by loading 5 μ l of the PCR amplification product on a 1.5% (w/v) agarose gel (Lonza) and electrophoresing this at 160V for 40 minutes. To assist with visualisation, ethidium bromide (0.5 μ g/ml, Sigma-Aldrich, St Louis, Missouri, USA) was added and the gel was photographed under UV illumination (Uvipro Gold, Uvitec, Cambridge, UK). The PCR products were visible and could be sized by comparison to the size ladder GeneRuler™ 100bp DNA ladder Plus (Fermentas, Hanover, Maryland, USA). The fragments were scrutinised to assess the amount of PCR product obtained and also whether it corresponded with the expected reference sequence size.

Direct cycle sequencing of GPC3 genomic regions

In order to obtain the best possible sequencing result, excess single stranded primers and dNTPs were removed from the amplified PCR products. An amount of 5 μ l of PCR product was combined with 0.2 μ l Exonuclease I (20 U/ μ l; Fermentas), 0.5 μ L FastAp (1 U/ μ l; Fermentas) and 4.3 μ l of distilled water and incubated on the GeneAmp 9700 PCR system (Applied Biosystems, Carlsbad, California, USA) at 37°C for one hour. The reaction ended with enzymatic inactivation at 72°C for 15 minutes.

Direct sequencing of the amplified *GPC3* exonic regions of both probands was undertaken. In direct cycle sequencing, primer sequences employed during amplification of DNA target template are used in conjunction with dNTPs and dideoxynucleotides (ddNTPs). The template is elongated by the addition of dNTPs and this elongation continues until a ddNTP is incorporated. The process is then promptly terminated as ddNTPs lack a 3' hydroxyl group for another dNTP to adhere to. During an automated sequencing process each ddNTP is differentially displayed due to its specific fluorophore label.

The sequencing reaction had a final reaction volume of 10µl. It consisted of 2µl of BigDye® Terminator v3.1 Cycle Sequencing mix (Applied Biosystems), 2µl of dilution buffer (Applied Biosystems) and 0.5µl of either a sense or antisense primer (at a final concentration of 0.4µM). Depending on the initial amplification yield, the amount of PCR product ranged from 3 - 5.5µl. The reaction was performed on the Bio-Rad T100™ thermal cycler (Bio-Rad) with initial denaturation of PCR products at 98°C for 5 min, followed by 35 cycles consisting of 96°C for 15 seconds, 55°C for 10 seconds and 60°C for 4 minutes.

To remove the unincorporated ddNTPs from the newly synthesised DNA fragments, 2.5µl of sodium acetate (pH5.2, 2.5M) and 30µl of absolute alcohol was added to the sequencing reaction mix. This was incubated overnight at -20 °C. Following the incubation reaction the sample was centrifuged for 10 minutes at 9300 rpm and the supernatant discarded. After the addition of 30µl of 70% ethanol, the sample was centrifuged for 10 minutes at 9300 rpm. Once the supernatant had been removed, the sample was air-dried. Sequencing for both probands and their respective mothers was performed on the ABI PRISM 3130xl Genetic Analyser (Applied Biosystem, HITACHI, Santa Clara, California, USA). The results of the sequencing reaction were analysed using SEQMAN (DNASTAR®, SeqMan™) software systems. The mutation found was investigated using two databases, the London Open Variation Database²¹ and the NCBI website²².

4.3 RESULTS

4.3.1 Phenotypic description of families B and S

4.3.1.1 *Proband B*

Proband B was evaluated by the investigator at the age of five years and nine months. He had previously been seen by the Division of Human Genetics of the University of Cape Town on two occasions - at birth, and at four years of age.

Family history

There was no family history of any intellectual concerns. There was a maternal cousin who had a cardiac lesion and bowel problems, but no further details were known. The boy is his mother's only child.

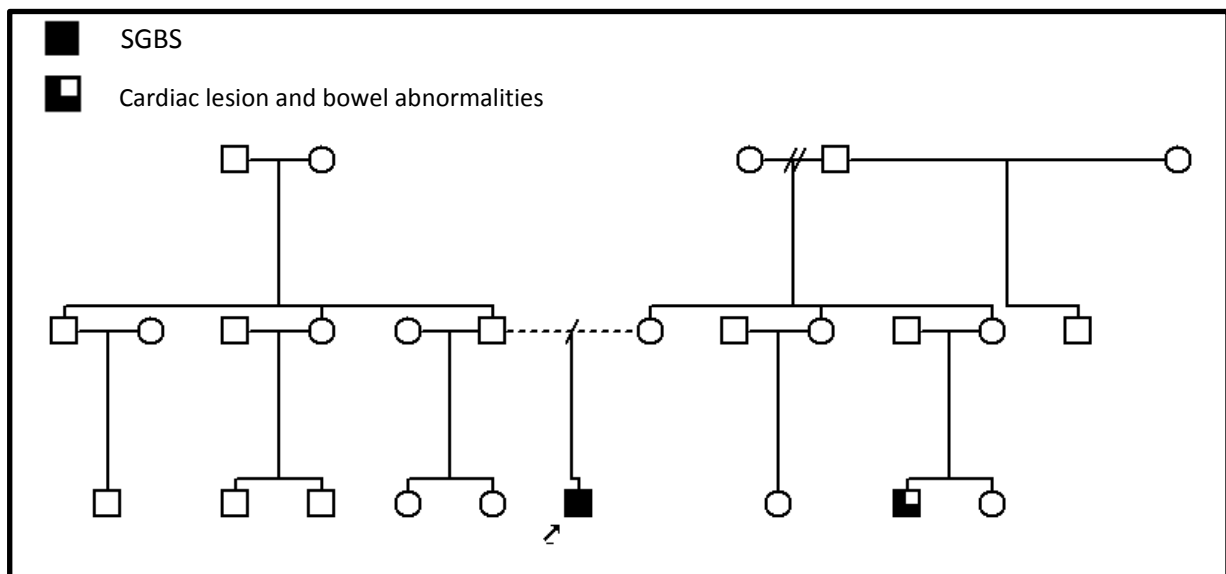


Figure 4.1: Pedigree of proband B's family.

Perinatal history

No exposure to known teratogens was reported during the pregnancy. No antenatal investigations were undertaken. The only complication during pregnancy was that of unexplained premature rupture of membranes at seven months.

Proband B was born by vaginal delivery in the breech position. His Apgar scores were recorded as 7 and 9. According to his mother he was born at seven months gestation. There

are no records documenting the gestation in weeks, which could have been anywhere from 28 to 32 weeks. His birth weight was 2.1kg. Consideration of the two extremes in gestational age in weeks would place him in a range from the 90th to above the 90th centile. His head circumference was 31cm, which is anywhere from above the 50th to above the 90th centile in this context. His length at birth was not documented.

He was admitted to neonatal ICU after birth and only discharged home after six weeks. He required ventilation for an aspiration pneumonia and also had prolonged jaundice. Notably he also had a single episode of hypoglycaemia after birth.

Clinical features

a) Growth parameters:

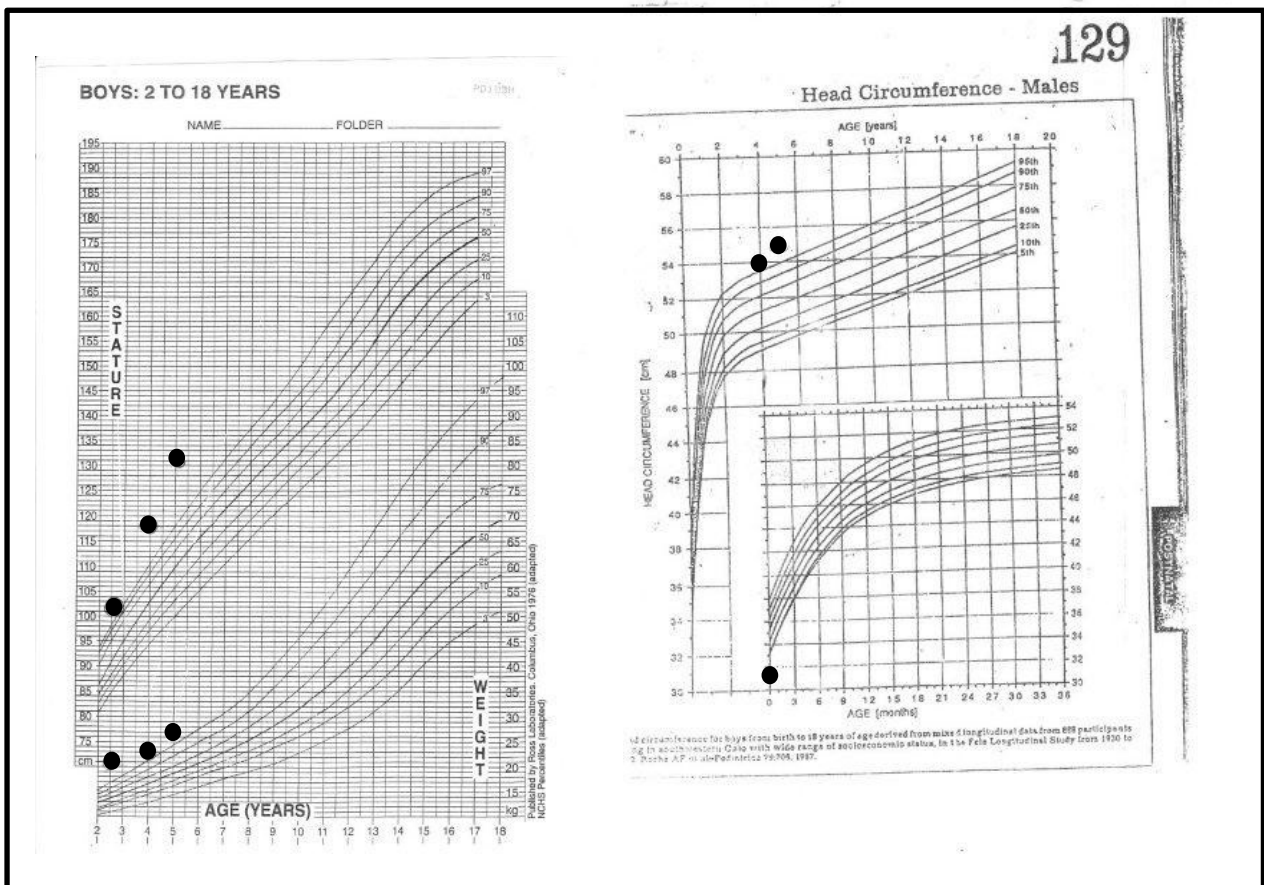


Figure 4.2: Growth parameters proband B. The weight, height and head circumference of proband B illustrating generalised overgrowth

b) Facial features

The boy had a long face, with hypertelorism, epicanthic folds and an upturned nose. His nasal bridge was broad. His mouth tended to be open and he had macrostomia and macroglossia with a grooved tongue and prognathism. His ears were normal.



Figure 4.3: A photograph of proband B. Hypertelorism, a broad nasal bridge and epicanthic folds are demonstrated. Macrostomia, macroglossia and a grooved tongue are evident in this image.

c) Skeletal system

Proband B had pectus excavatum and a marked lordosis. No obvious scoliosis was noted. There was no polydactyly or any other obvious hand abnormalities. He did not have a cleft lip or palate. On chest X-ray there were 13 ribs noted on each side.



Figure 4.4: A full length image of proband B. Proband B had a marked pectus excavatum and a protruding abdomen.

d) Cardiovascular system

He had a soft ejection systolic murmur with no signs of cardiac failure. Cardiovascular investigations revealed an atrioseptal defect (ASD) and valvular pulmonary stenosis.

e) Genito-urinary system

He had had bilateral inguinal hernias repaired. No other hernias were documented. He had hypospadias and a shawl scrotum.

f) Abdominal system

Abdominal examination revealed a large mass in the left flank which raised suspicion of neoplasia. No other organomegaly or mass was detected. Although he had received treatment for constipation on several occasions, there had not been any concern of intestinal malformations noted in his medical records.

g) Neurological involvement

Formal central nervous system imaging had not been undertaken. His developmental milestones had all been within the normal range but he had been noted to be hypotonic. At five years his speech is still slightly difficult to comprehend although his vocabulary was good. The rest of his development was appropriate for his age.

There were no focal neurological abnormalities.

h) Tumours

He was diagnosed with a Wilms tumour of his left kidney at the age of five years and nine months.

Clinical course and management:

At two months of age proband B had an apnoeic attack and was admitted to the neonatal ICU.

It was during this time that ambiguous genitalia were noted. A diagnosis of a hypospadias with a penoscrotal meatus and chordee was made. He was treated as an undervirilised male, with possible partial androgen insensitivity syndrome.

At three months of age the boy had a bilateral inguinal hernia repair and a right orchidopexy. He had a first stage hypospadias repair at almost five years of age. A year later he was investigated for a mass in his left flank. A Wilms tumour was diagnosed. He was started on chemotherapy and the tumour responded well. After chemotherapy he had a partial left nephrectomy. His latest imaging revealed no residual tumour or recurrence.

During investigations for the tumour he was also found to have right hydronephrosis secondary to a pelvo-ureteric obstruction. The obstruction was relieved by inserting a stent. Although the kidney remained hydronephrotic, renal function has shown great improvement. The obstruction is still present despite a pyeloplasty.

He is still under the care of oncologists and urologists undergoing standard tumour surveillance¹ (Table 4.3) and is awaiting final repair of his hypospadias. His cardiac lesion does not need intervention.

Table 4.3: Recommended tumour surveillance program¹

	Physical examination	Abdominal ultrasound	Alpha feto protein and βHCG	Urinary catecholamines, VMA and HVA	Chest Xray
0-4 years	3 monthly	3-4 monthly	4 monthly	4 monthly	Annually
4-7 years	4 monthly	3-4 monthly	6 monthly	6 monthly	Annually
>7 years	Biannually	Yearly	Annually	Annually	Annually

Investigations

Following the early diagnosis of ambiguous genitalia proband B had extensive endocrine investigations:

- A karyotype revealed a normal male pattern. 46,XY.
- Oestradiol, Follicular stimulating hormone (FSH) and Luteinising hormone (LH) levels were normal
- Dehydroepiandrosterone (DHEAS) and 17-hydroxyprogesterone (17-OHP) were mildly raised but not considered to be a concern.
- Adequate testosterone response with β HCG stimulation test.
- Pelvic ultrasound revealed no Mullerian structures and two testes were seen.
- 5 α -reductase deficiency was excluded.

Cardiology:

- Atrioseptal defect with valvular pulmonary stenosis

Investigations of Wilms tumour

- Histology obtained from a fine-needle aspiration confirmed a nephroblastoma.
- An MRI of his chest showed metastatic spread to the left lung base.

Description of mother of proband B

Proband B's mother was a healthy woman. She had jaundice as a child but no other neonatal concerns. She was diagnosed with scoliosis which resolved spontaneously during her teens. She was tall, and at 1.81 cm plotted above the 97th centile. She had a long face with no other obvious dysmorphic features. She did not have any learning difficulties.



Figure 4.5: Proband B and his mother. Proband B's mother had tall stature and a long face.

4.3.1.2 *Proband S*

Proband S was evaluated at four years and two months of age. He had been followed up for a prolonged period at the genetic clinic at Red Cross War Memorial Children's Hospital.

Family history

There was a family history of mild intellectual disability, which is indicated in the pedigree.

There was no family history of congenital malformations

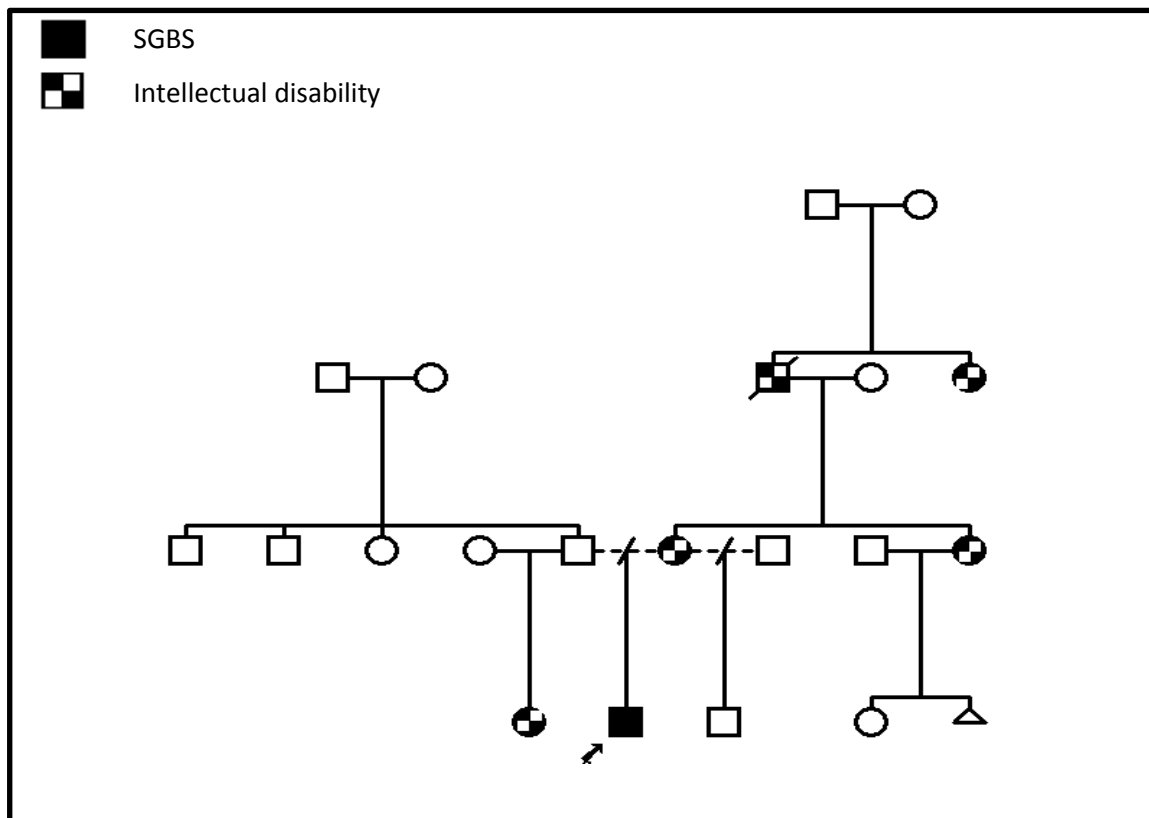


Figure 4.6: Pedigree of proband S's family. There is a family history of intellectual disability

Perinatal history

His mother has asthma, but was otherwise in good health during the pregnancy. There was no history of teratogen exposure. Ultrasound investigations during the mother's pregnancy did not reveal any abnormalities.

Proband S was born via normal vertex delivery at term. He had Apgar scores of 8 and 9. His birth weight was 3.2 kg (10th centile). The birth head circumference was 33cm (5th centile).

After birth he was admitted to the neonatal ICU following an apnoeic episode and he received assisted ventilation for two weeks. This illness was presumed to be due to unconfirmed sepsis and he also had jaundice which necessitated phototherapy. There were no hypoglycaemic events.

Clinical features

a) Growth parameters

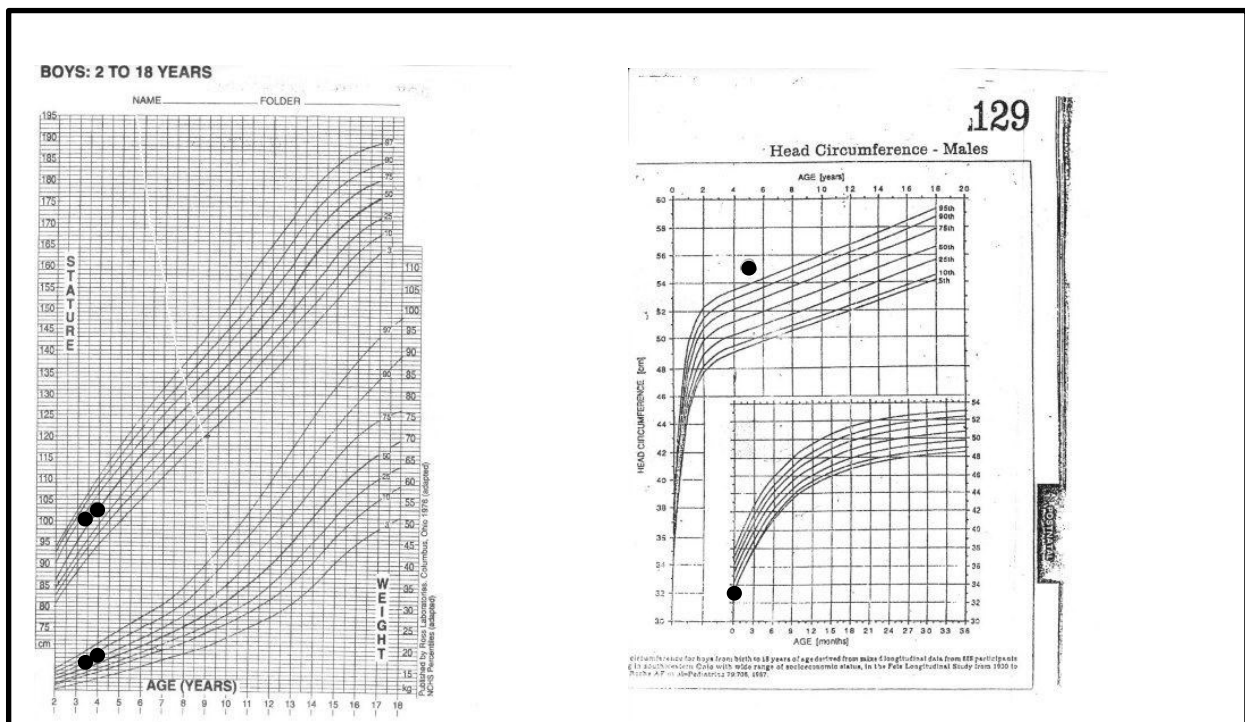


Figure 4.7: Growth parameters of proband S. Head circumference is well above the 95th centile

b) Facial features

The boy had a coarse face with hypertelorism, downslanted palpebral fissures, epicanthic folds and an upturned nose. His tongue was protruding, macroglossic, and had a central groove. He also had macrostomia. He was mildly hirsute with a low posterior hair line. His ears were normal.

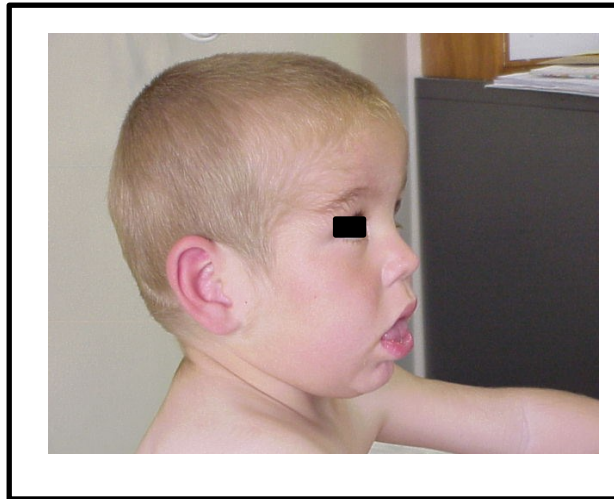


Figure 4.8: A profile photograph of proband S. There is hirsutism of his forehead



Figure 4.9: A photograph of proband S. The features evident in this photograph are a coarse face, hypertelorism, epicanthic folds and an upturned nose. There is also macroglossia with a grooved tongue

c) Skeletal

Proband S had broad, short fingers with bilateral fifth finger clinodactyly. His toes were broad and he had pes planus. He also had a mild lordosis causing his stomach to appear to be protruding. There was no evidence of a cleft lip or palate.

d) Cardiology

The boy had a patent ductus arteriosus with a patent foramen ovale. This was evaluated by cardiologist and found to have closed spontaneously.

e) Genito-urinary system

His genitalia were normal.

f) Abdominal system

He had hepatomegaly of 2cm below the costal margin. This feature had been stable for a number of years.

g) Neurological abnormalities

He was hypotonic, with no focal neurological deficit. He had been assessed by a developmental specialist and was found to have a developmental quotient of 60, in keeping with mild intellectual disability.

h) Tumours

At the time of this research he had not developed any tumours.

Clinical course and management

During the neonatal period proband S had very loose skin and a diagnosis of cutis laxa was considered and later abandoned.

From six months onward his head circumference showed a steady centile increase when compared to his other growth parameters. A metabolic work-up was undertaken during this time and no abnormalities were detected.

He is currently undergoing tumour surveillance according to the recommended protocol (Table 4.3).

He is attending a special educational needs school.

Investigations

A Karyotype revealed a normal male pattern. 46,XY.

Thyroid function was normal

A metabolic work-up included the following tests

- Plasma and urine amino-acid profiles were normal
- Urine organic acid profile was normal
- No urine reducing substances

An investigation into storage disorders was also undertaken:

- Urinary glycosaminoglycans was normal
- Leucocyte vacuolation was normal
- Normal alpha glucosidase

A skin biopsy in the neonatal period evaluating elastin and collagen showed non-specific changes

A chest X-ray revealed 11 ribs on the right and 12 on the left.

On ultrasound of his abdomen, the boy had homogeneous mild hepatomegaly with no other abnormality. His kidneys were reported as normal.

An MRI of his brain at two years of age showed atrophy and a peritrigonal high signal which were suggestive of a hypoxic insult at birth. No other specific abnormalities were found.

Description of mother of proband S

Proband S's mother had a mild intellectual disability and attended a school for learners with special educational needs. She was functionally independent and employed. Her height was 176cm which is above the 97th centile. She had a long face, with a high arched palate. The other family members with intellectual disability were not available for examination.

4.3.1.3 Comparison of clinical features

There are no diagnostic criteria available to assist in the clinical diagnosis of SGBS. There are however, some clinical features that would be suggestive of a diagnosis of SGBS if they are present¹. Table 4.4 and 4.5 compare proband B and S to common features and malformations which have been documented in SGBS.

Table 4.4: Comparison of the facial features of probands B and S to common features documented in SGBS

Craniofacial features¹	Proband B	Proband S
Macrocephaly	Yes	Yes
Hypertelorism, epicanthic folds, downslanted palpebral fissures	Yes	Yes
Redundant skin over glabella	No	No
Macrostomia	Yes	Yes
Macroglossia	Yes	Yes
Midline groove lower lip or tongue	Yes	Yes
Cleft lip or palate or high, narrow palate	No	No
Macrognathia	Yes	No

Table 4.5: Comparison of the associated features in probands B and S compared with those frequently described in SGBS

Associated features¹	Proband B	Proband S
Macrosomia	Yes	No
Congenital heart disease	Yes	Yes
Conduction defects	No	No
Supernumerary nipples	No	No
Diastasis recti/umbilical hernia	No	No
Diaphragmatic hernia	No	No
Renal dysplasia/nephromegaly	Yes	No
Cryptorchidism/hypospadias	Yes	No
Hand anomalies	No	Yes
Rib abnormalities	Yes	Yes

4.3.2 Molecular analysis of families B and S

The molecular results of both probands B and S are presented together under this heading. This format is used for the sake of expediency in the light of the negative findings in proband S.

4.3.2.1 DNA extraction

The spectrophotometry of the DNA obtained from all samples revealed results that were suitable for analysis. The extracted DNA also showed a good yield on gel electrophoresis (Lonza).

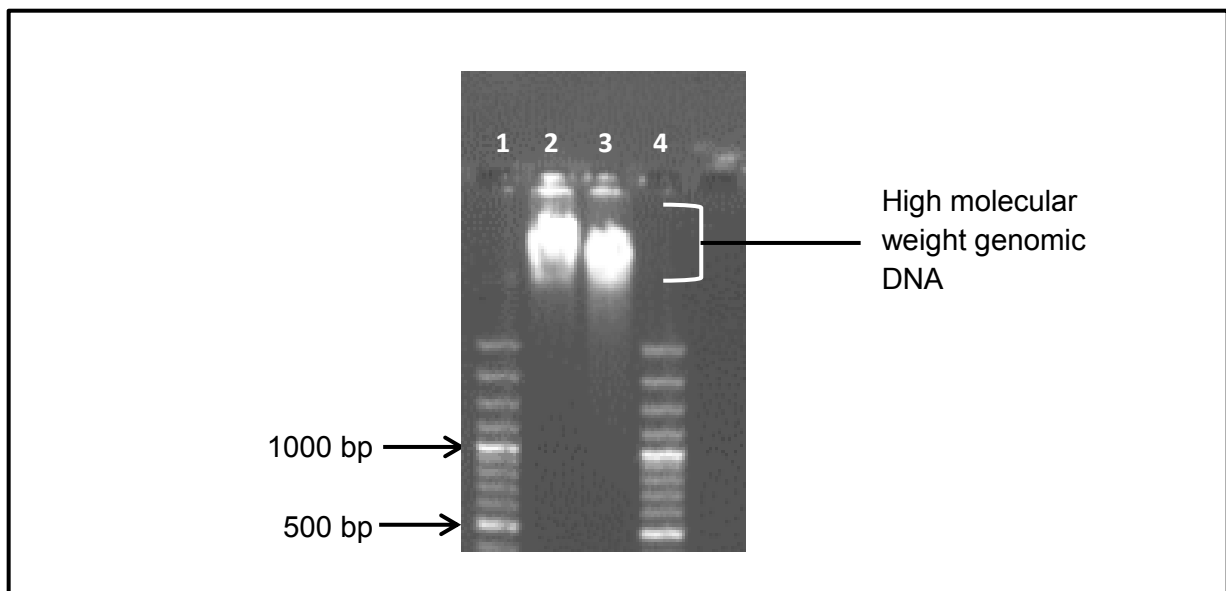


Figure 4.10: An image of a 1.5% agarose gel for the assessment of genomic DNA integrity. Lanes 1 and 4 contain 500ng of Generuler 100bp Plus (Fermentas), lanes 2 and 3 contain 200ng of Proband B and his mother's extracted genomic DNA, respectively.

4.3.2.2 PCR amplification

In order to assess whether all the *GPC3* exon regions amplified during the PCR reaction the products were scrutinised for specificity and amplification yield for downstream applications by electrophoresis of 5µl of the product on a 1.5% agarose gel. All the fragments in both probands, as well as their mothers, were successfully amplified and were visible on the agarose gel. This not only indicated that the PCR amplification process was successful but also that there were no deletions present resulting in altered banding patterns between the probands and their mothers. Figure 4.11 illustrates the results of the four participants.

4.3.2.3 Sequencing reaction

Proband B:

The entire coding region, including intron-exon boundaries of *GPC3*, was analysed by direct DNA sequencing.

A mutation was found in exon 4 of proband B. This mutation consists of a deletion of four nucleotides, TAGA, at nucleotide position 1071, and an insertion of three nucleotides, CTT. This mutation is designated c.1071delTAGAinsCTT. This alteration causes a frameshift, which results in a premature stop codon. Figure 4.12 illustrates this mutation.

At a protein level this tetranucleotide deletion results in a disruption of the amino acid reading frame which causes Arginine to be replaced by Phenylalanine at position 358, with a resulting premature stop codon at position 373. Using Genbank accession no. NM_004484.3 as a reference, the mutation can be described as p.358Arg-PheFSX373. As this premature stop codon occurs in exon 4, the majority of the coding sequence of *GPC3* is not translated.

This mutation was confirmed to be present in proband B's mother in the heterozygous state.

Proband S:

Sequencing results of all exons of *GPC3* were analysed and no variation when compared to the reference sequence, was found in either probands S or his mother.

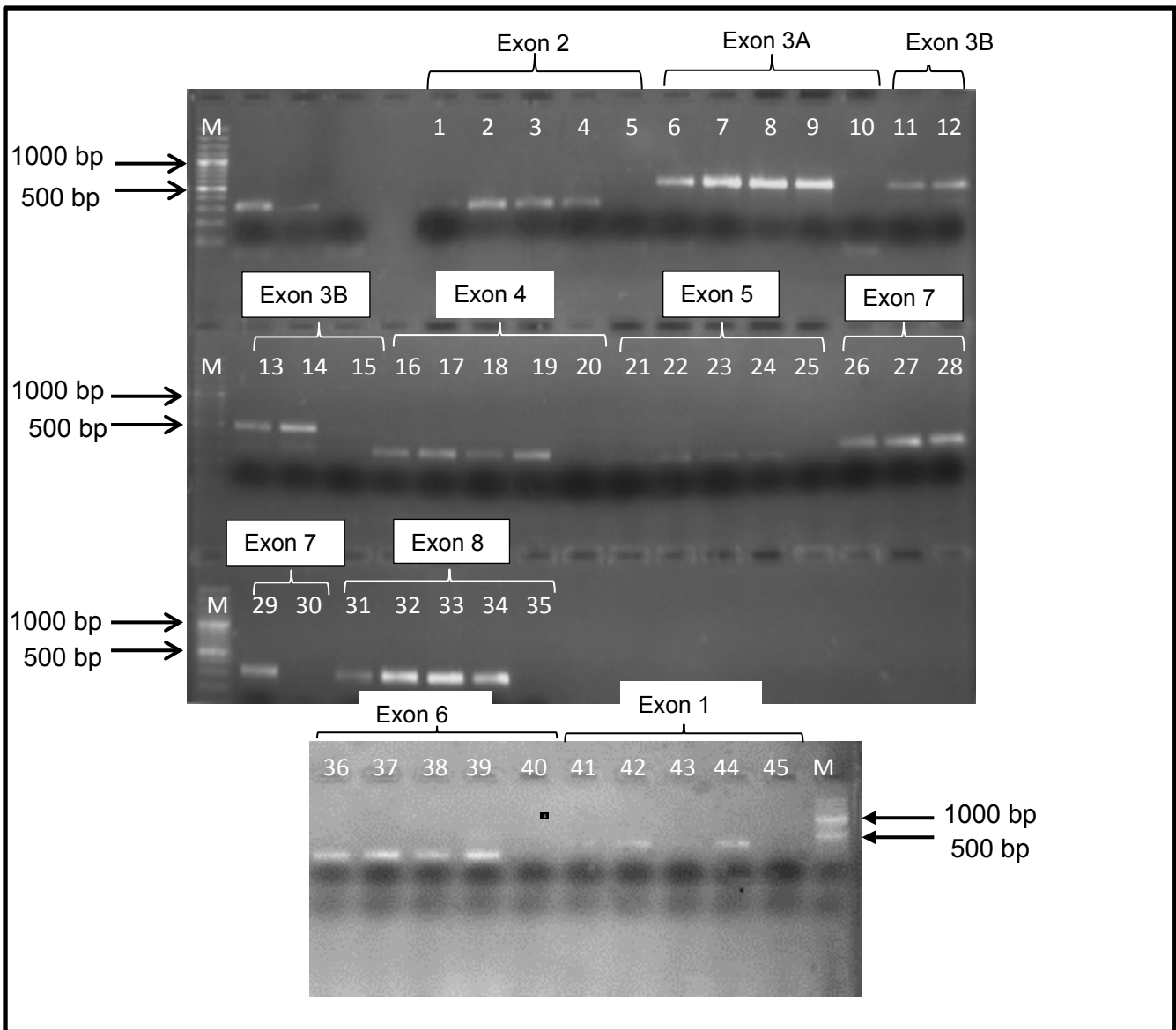


Figure 4.11: An image depicting the agarose gel electrophoreses of amplified PCR products for all four participants. M contains the 500ng of GeneRuler 100bp Plus (Fermentas)

Exon 2, 1=proband B, 2=proband B's mother, 3=proband S, 4=proband S's mother, 5=no-template control. Expected size=277 bp

Exon 3A, 6=proband B, 7=proband B's mother, 8=proband S, 9=proband S's mother, 10=no-template control. Expected size=493 bp

Exon 3B, 11=proband B, 12= proband B's mother, 13=proband S, 14=proband S's mother, 15=no-template control. Expected size=501 bp

Exon 4, 16=proband B, 17= proband B's mother, 18=proband S, 19=proband S's mother, 20=no-template control. Expected size=302 bp

Exon 5, 21=proband B, 22= proband B's mother, 23=proband S, 24=proband S's mother, 25=no-template control. Expected size=267 bp

Exon 7, 26=proband B, 27= proband B's mother, 28=proband S, 29=proband S's mother, 30=no-template control. Exoected size=327 bp

Exon 8, 31=proband B, 32 = proband B's mother, 33=proband S, 34=proband S's mother, 35=no-template control. Expected size=306 bp

Exon 6, 36=proband B, 37 = proband B's mother, 38=proband S, 39=proband S's mother, 40=no-template control. Expected size=255 bp

Exon 1, 41=proband B, 42 = proband B's mother, 43=proband S, 44=proband S's mother, 45=no-template control. Expected size=399 bp

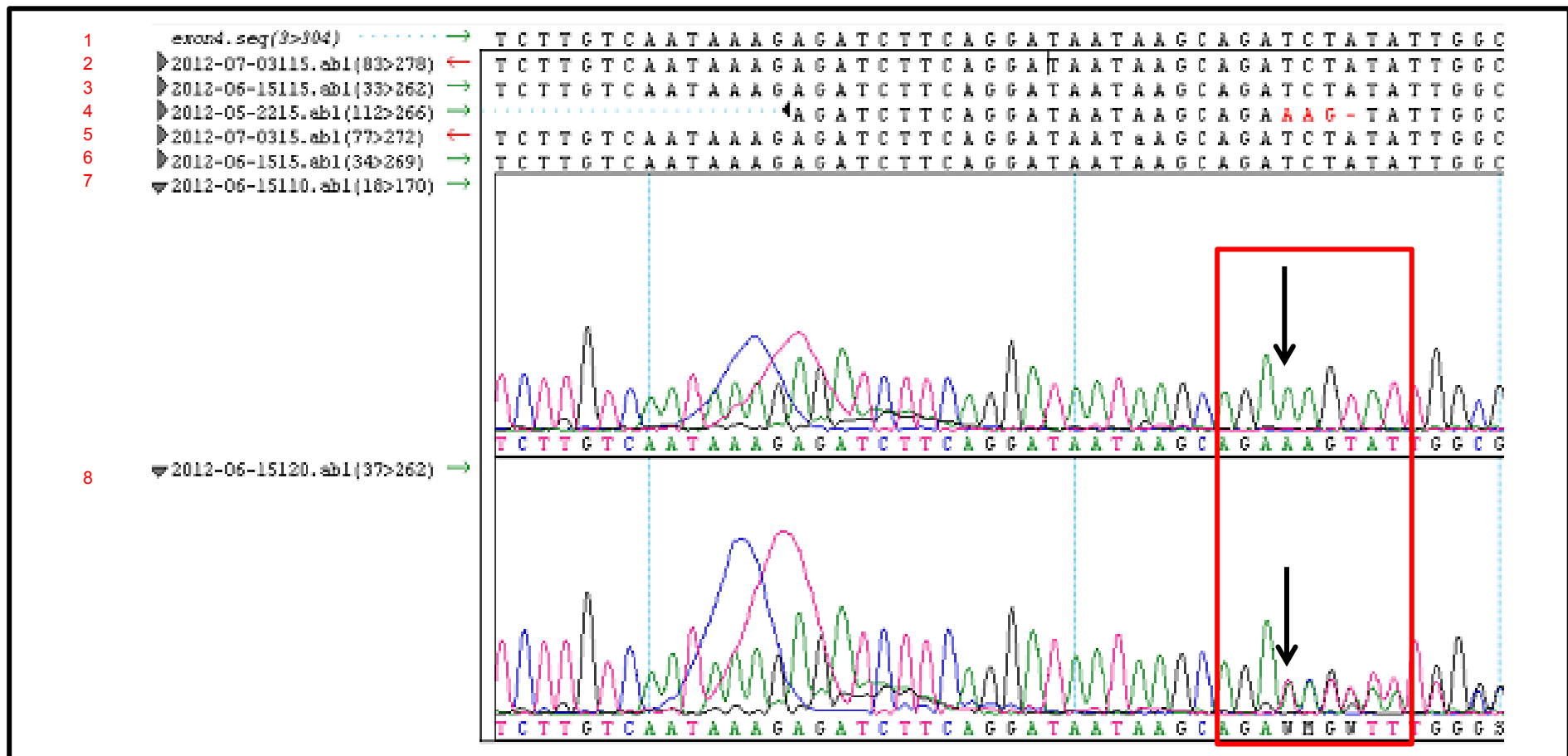


Figure 4.12: An electropherogram of proband B and his mother illustrating the mutation detected in exon 4 of the GPC3 gene. The sequencing reaction was analysed using DNASTAR SeqMan. Label 1 is the reference sequence. Label 2 and 3 is the sequencing result of exon 4 of proband S's mother revealing no mutations. Label 5 and 6 is the normal sequencing result of exon 4 in proband S. Label 4 and 7 indicate the sequencing result of exon 4 in proband B, the arrow indicates the beginning of the TCTA deletion, and insertion of AAG (the annotation used in the text is that of the complementary strand, i.e. deletion TAGA and insertion of CTT). Label 8 is the results of proband B's mother, her results reveal the same mutation as her son, in a heterozygous state

4.4 DISCUSSION

4.4.1 Phenotype

There are no published data describing SGBS in South Africa. The boys who were included in this investigation exhibited many of the more common features described in SGBS. A limitation of this study is that both of the probands investigated were of Caucasian ancestry. This is not representative of the diverse populations groups in South Africa, however there is no indication in the literature reviewed for this research that differences in presentation would be expected^{3,13}. More information regarding phenotypes in other ethnic groups in South Africa would have been valuable.

There are ultrasound abnormalities that can lead to consideration of an antenatal diagnosis of SGBS. These are macrosomia, an increased nuchal translucency, congenital diaphragmatic hernia, visceromegaly, and renal abnormalities. Polyhydramnios and an elevated maternal serum alpha fetoprotein (msAFP) in addition to the above anomalies warrant consideration of SGBS as a diagnosis²³. In South Africa there is limited access to detailed fetal anomaly scans and this information will be useful mainly in tertiary centres where this service is available. The msAFP investigation is offered routinely in the private sector in South Africa and although an elevation in this value is seen in pregnancies affected with SGBS, an increased msAFP may indicate of many other conditions, including neural tube defects²⁴.

Antenatal ultrasound investigations were only undertaken in proband S. These findings were normal. An antenatal diagnosis of SGBS was not considered in either of our probands. Both probands' mothers will, however, be offered detailed fetal ultrasound monitoring during future pregnancies.

Proband B had been macrosomic since birth. Proband S had a weight above the 75th centile, and a head circumference above the 97th centile. His height was around the 50th centile. Although not strictly fulfilling the definition of macrosomia, he showed an element of postnatal overgrowth by acceleration of his growth parameters and evolution of head circumference.

Both probands had facial features consistent with those described in the literature. They both had hypertelorism, an upturned nose and macrostomia. These features are often described in individuals affected with SGBS⁶. Both boys also had macroglossia with a midline groove in the tongue. Macroglossia is seen in 40% of individuals with SGBS⁷. In addition proband B

had a prominent jaw and a broad nasal root which is also frequently described⁶. Proband S has a coarse face, and is also hirsute. Hirsutism is not typically a feature of SGBS, but a coarse facies has been reported in 83% of persons with SGBS⁷. Both proband B and S had epicanthic folds, which is a classical, although non-specific characteristic of SGBS²⁵.

Some of the typical skeletal features previously described were also seen in the boys included in this research. Both had numerical rib abnormalities and lordosis. Proband B had pectus excavatum, and proband S had short broad hands – both are frequent skeletal manifestations of SGBS⁶. Notably neither polydactyly nor palatal clefting were present in these individuals.

The boys both had structural cardiac lesions which did not require any surgical intervention. These structural malformations, an atrioseptal defect (ASD) and a patent ductus arteriosus (PDA), have previously been described in SGBS¹⁰.

Hydronephrosis and inguinal hernias are commonly described in the SGBS, while hypospadias has less frequently been documented⁹. The severity of the genito-urinary abnormalities documented in proband B has been reported in other individuals with SGBS⁹.

Visceromegaly is reported as a common feature and this would be in keeping with the non-progressive hepatomegaly in proband S⁶. Proband S did not have ear lobe creases, an abdominal wall defect or other clinical features to suggest Beckwith Wiedeman. His facial features were also not in keeping with the bossed forehead or prominent chin described in Sotos syndrome.

The variability in intellectual outcome is also evident in these boys, with one showing mild intellectual disability and the other appearing to develop normally. Proband S has a family history of mild intellectual disability. His mother and his maternal aunt are similarly affected and his maternal grandfather and great-aunt were reported to have intellectual difficulties too. There are two possible explanations for this.

Firstly, the familial intellectual disability can be explained by SGBS. This feature has been described in many males with SGBS. With respect to the affected females, their intellectual disability could be explained by SGBS carrier status. There have been reports of female carriers of SGBS exhibiting mild manifestations of the condition. A female with an X-autosome translocation causing skewed X-inactivation has previously been shown to have typical features of SGBS. Furthermore there has been a report of a girl with developmental delay and dysmorphic features strongly suggestive of SGBS. She was heterozygous for a mutation in *GPC3*. Two of her brothers clinically affected with SGBS had a similar mutation.

X-inactivation studies revealed a pattern of inactivation moderately skewed toward her normal X-chromosome (71-80% inactivation of her normal allele). Her mother was of normal intelligence and had only mild clinical signs of SGBS. Her mother's inactivation studies showed much lower levels of inactivation of her unaffected allele. This led the authors to postulate that skewed X-inactivation was the cause of her phenotype and that there appears to be a minimal threshold value of X-inactivation at which clinical features will be manifested²⁶. It is thus plausible that proband S's mother could have skewed X-inactivation surpassing this threshold causing her to have intellectual disability, tall stature and mild facial dysmorphic features. All the females with intellectual difficulties are connected to proband S via an X-linked inheritance pattern and therefore the same could be true for them. In the case of proband S's maternal grandfather, there is a possibility that he could have had SGBS himself, causing both his daughters to be obligate carriers of the condition. Studies for skewing of X-inactivation were beyond the scope of this research but could have provided useful information.

Secondly, the cause of the intellectual disability in either proband S, or any of his similarly affected family members, may be multifactorial or related to other genetic factors. Although the inheritance pattern in this family is compatible with X-linked inheritance it is not the only method of inheritance plausible. Unfortunately the boy's aunt was not available for examination, and his maternal grandfather has passed away so it was not possible to further investigate the cause of their intellectual disability.

Proband S had a normal karyotype. A karyotype was not performed on his mother.

Proband B developed a Wilms tumour. This tumour has been described in at least four other affected individuals and the association between Wilms tumours and SGBS is well established. Overall, the literature reports 10 instances in which persons with SGBS developed a tumour¹¹, the proband in this research represents an 11th case. The affected boy has recovered well and he will continue to receive tumour surveillance in his hometown.

Both boys included in this study had some typical and associated features of SGBS. Their manifestations are consistent with those described in the literature. The diagnosis is not easy to make and there is considerable overlap with the other syndromes. Equally, the facial features may not be recognisable in smaller babies. Both probands were diagnosed in early childhood. The main clinical manifestations which prompted this diagnosis in the two boys were macrosomia, coarse facial features, macroglossia and a grooved tongue.

4.4.2 Molecular analysis

Many different mutations in *GPC3* have been described as causative in the SGBS. No hotspots for these mutations have been identified but frameshift mutations and deletions have been reported on numerous occasions^{6,14,17}. All mutations associated with SGBS either remove a start codon or introduce a premature stop codon. These premature stop codons invariably lead to a truncated protein with insufficient cysteine residues in the conserved cysteine motif. This motif is thought to be important for the tertiary structure of glypicans¹⁵. These abnormal proteins will also not contain the site for heparan sulfate attachment, causing failure of heparan sulfate substitution.

Although the specific mutation found in proband B has not previously been reported, its effects appear to be similar to other mutations associated with SGBS. The sequence anomaly disrupts the reading frame and introduces a premature stop codon, resulting in the majority of the protein not being translated.

Two databases were investigated to evaluate whether the mutation in Proband B has been previously reported to be associated with SGBS. The Leiden Open Variation database²¹ did not contain any mutations in *GPC3* and although the NCBI website²² had many known mutations the above mutation has not been previously reported. This indicates that this is a novel mutation.

Proband B's mother was found to be heterozygous for this mutation. Accurate counselling regarding the recurrence risk for future pregnancies is now possible. In any future pregnancy, a male fetus will have a 50% chance of being affected with SGBS. A female fetus will have a 50% chance of being a carrier of this mutation.

The research results were delivered to proband B's mother during a genetic counselling session. Antenatal diagnosis will be available to her should she want to pursue this option, following confirmation of this mutation in a diagnostic laboratory. This testing could be carried out as a prenatal diagnosis through a chorionic villous biopsy or amniocentesis. Preimplantation genetic diagnosis could also be used to prevent another affected pregnancy, this is however an expensive procedure and is not routinely available to patients using government facilities.

If the decision is made not to undergo any prenatal testing and a pregnancy is conceived naturally, testing of a male infant could be offered to the mother. Testing of a minor would be ethically justified in this case as the result would have a direct impact on his medical management. Should this potential sibling be affected with SGBS, early initiation of a tumour

surveillance program may be considered. The manifestations of SGBS can be subtle, especially in infants, and a clinical diagnosis may only be evident at a later stage. This could lead to a delay in monitoring for tumour development. Cascade screening following a genetic counselling process has also been offered to this family.

No mutation was identified in proband S even though mutations in *GPC3* are present in up to 70% of individuals with SGBS¹. There are several possible reasons for this. The first possibility is that in proband S, SGBS is caused by a mutation in a gene other than *GPC3*. The likely other candidate gene is *GPC4*, although there has been only one report of a duplication in *GPC4* without mutations in *GPC3*¹⁶. No other candidate genes have been reported but a number of clinically affected boys have not yet had the molecular basis of their SGBS identified, raising the possibility of additional genes being implicated.

A second possible reason that a mutation was not found could be because it is in the intronic region of *GPC3*. The primers used in this study were designed to encompass intron exon boundaries and also a significant part of the introns. This makes it less likely that there is a mutation in the introns that will affect the splice site and cause a disruption in the protein.

Finally it is possible that proband S does not have SGBS. SGBS is a difficult clinical diagnosis to make as it has significant phenotypic overlap with other overgrowth syndromes. As the clinical phenotype described in proband S is consistent with previous reports of SGBS, this is a less likely scenario. For this reason he will still continue with his tumour surveillance program.

4.5 CONCLUSION

This report represents the first published description of South African patients with SGBS. This condition is a recently described, under-diagnosed syndrome. The implications of a diagnosis of SGBS do not only relate to the clinical diagnosis and recurrence risk in siblings, but also to the predisposition for developing tumours.

In this research, clinical features in two probands from two unrelated families were documented. The phenotype of the boys included in this research is similar to that previously reported in the literature. On this basis clinicians in South Africa can be guided by the literature in the diagnosis of SGBS. There are no diagnostic criteria but in this report an attempt was made to highlight the more common clinical findings. Macrosomia and typical facial features associated with other malformations in the skeletal, cardiovascular, renal or uro-genital systems are useful clinical indicators. Consistent manifestations amongst the two

probands in this study were their facial features and the element of overgrowth. The associated abnormalities and malformations were varied, although both had cardiac involvement. The importance of regular tumour surveillance is reinforced in this research by the documentation of the Wilms tumour that proband B developed.

An X-linked inheritance pattern can be used to distinguish SGBS from the other overgrowth syndromes as this condition is the only overgrowth syndrome known to exhibit this form of genetic transmission. The pedigree in family S could be consistent with X-linked inheritance.

The mutation found in proband B represents a novel, and likely disease-causing mutation. The literature supports this statement as the functional effect of this mutation, a premature stop codon, has been described numerous times. Identification of the mutation in this family presents the possibility of antenatal testing for the mother in future pregnancies, as well as testing of other at risk relatives in this family if diagnostically confirmed.

No mutation was identified in proband S. The possible reasons for this are discussed under the previous heading. Further investigation of *GPC4* and also dosage analysis of *GPC3* could help to make a diagnosis and will be considered in the future.

Routine molecular testing for SGBS is currently unavailable in South Africa. It is hoped however, that this research will benefit other children in South Africa as testing can now be offered on a research basis. If there is a demand for testing it could also be converted into a diagnostic test using the methods and analysis optimised in this project. Testing for X-linked conditions such as SGBS is important as it offers the possibility of carrier testing which could have implications for extended family members. SGBS is also associated with a risk of developing embryonal tumours, confirmation or exclusion of a clinical diagnosis would then also indicate whether an individual needs to continue with further tumour surveillance or not.

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5 APPENDICES

5.1 Appendix 1

Name:				
Date of birth and folder number:				
Pedigree:	Sex:	Age:		
Antenatal history:				
Antenatal ultrasounds:				
Delivery:	Where:	Gestation:	Method:	Apgars
Postnatal complications:				
Birth parameters:	Weight (kg):	COH (cm):	Length (cm):	
Post natal parameters	Weight (kg):	COH (cm):	Height (cm):	
Facial features:	Coarse facies <input type="checkbox"/> Other <input type="checkbox"/> explain: Hypertelorism <input type="checkbox"/> Downslanted palpebral fissures <input type="checkbox"/> Upturned nose <input type="checkbox"/> Macrostomia <input type="checkbox"/> Macroglossia <input type="checkbox"/> Grooved tongue <input type="checkbox"/> High arched palate <input type="checkbox"/> Cleft lip/palate <input type="checkbox"/> Prominent jaw <input type="checkbox"/>			
Thorax:	Pectus deformities <input type="checkbox"/> Supernumerary nipples <input type="checkbox"/> Other <input type="checkbox"/> explain:			
Cardiovascular system:	Cardiac murmur <input type="checkbox"/> Echography report explain:			

Abdominal:	Umbilical hernia <input type="checkbox"/> Diaphragmatic hernia Gastrointestinal obstructions <input type="checkbox"/> Organomegaly <input type="checkbox"/> Masses felt <input type="checkbox"/> explain:			
Limbs:	Hand polydactyly <input type="checkbox"/> Hand syndactyly <input type="checkbox"/> Blunt finger tips <input type="checkbox"/> Hypoplastic /dysplastic nails <input type="checkbox"/> Broad, short fingers <input type="checkbox"/>		Toe syndactyly <input type="checkbox"/> Broad toes <input type="checkbox"/> Clinodactyly <input type="checkbox"/> Other <input type="checkbox"/> explain:	
Genitourinary abnormalities:				
Skeletal abnormalities:				
CNS abnormalities:				
Development milestones:	Smile:	Sat:	Walk:	Talk:
Development (months):	Gross motor:	Fine motor:	Social:	Speech:
Investigations done:	Genetic tests:		Other:	
Tumour surveillance	Ultrasounds done:		AFP done:	
Tumours found (age):				

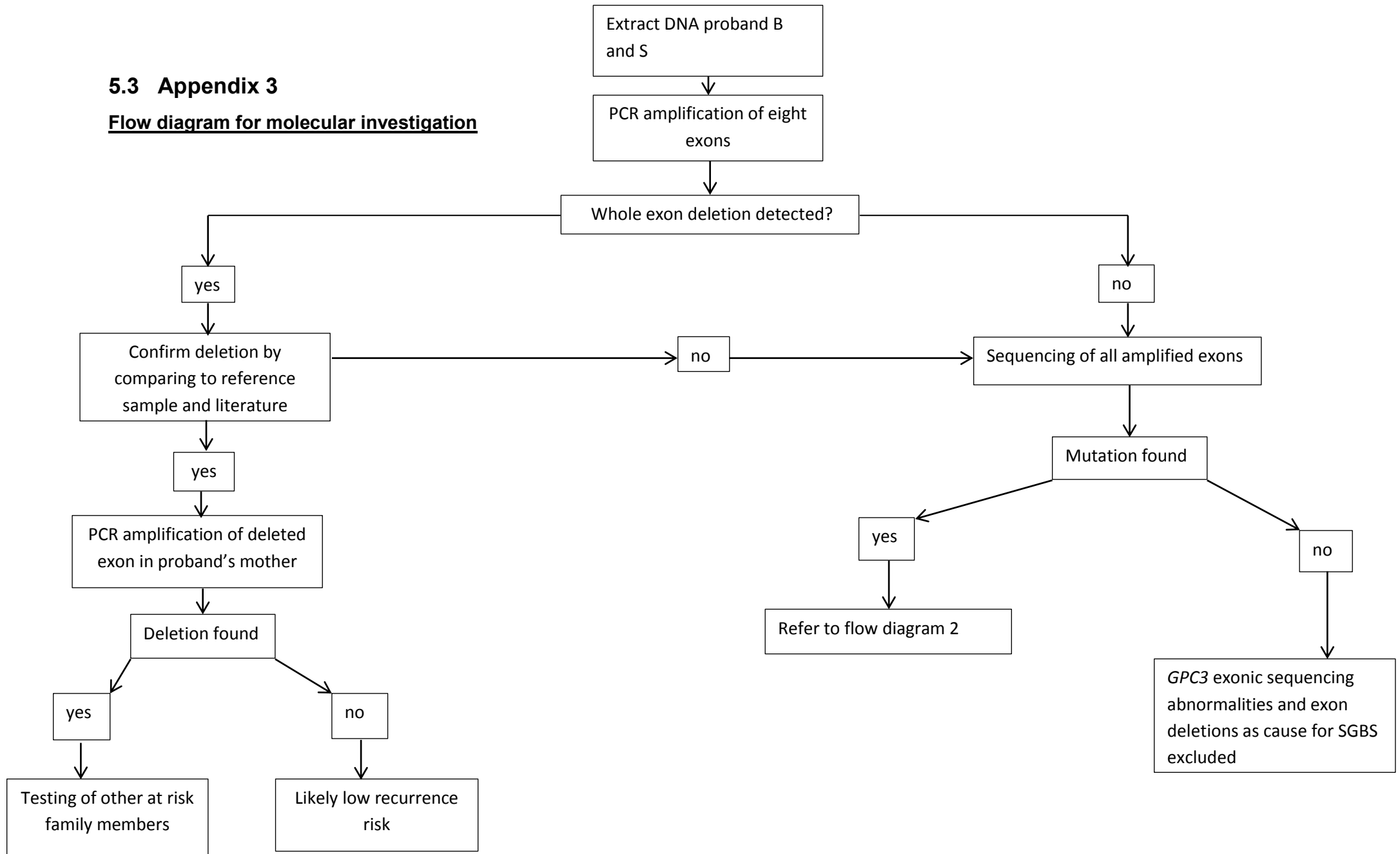
5.2 Appendix 2

Additional description for information needed on datasheet for clinical examination:

1. Name: Name of patient
2. Date of birth and folder number: Date of birth and folder number.
3. Pedigree: Refer to family's pedigree and enter pedigree number
4. Sex: Male or female
5. Age: For adults in years, for children in months
6. Antenatal history: Document any problems during the pregnancy. Specifically relating to the following:
 - Use of teratogens
 - Diabetes
 - Medications used
7. Antenatal ultrasound: Document at which gestational age and short report on what was seen. If known, comment specifically on poly/oligohydramnios
8. Delivery: State clearly at which hospital and the type of delivery
9. Postnatal complications: Noting any prolonged neonatal stay, ventilation, or problems maintaining glucose.
10. Birth parameters: As recorded on Road to Health card
11. Post natal parameters: Documented the current/last measurements taken.
12. Facial features: Refer to tick sheet
13. Thorax: Refer to tick sheet
14. Cardiovascular system: Refer to tick sheet
15. Abdominal: Refer to tick sheet
16. Limbs: Refer to tick sheet
17. Genitourinary system: note any abnormality and relevant times of surgery and follow up. Also comment on renal ultrasounds that were done
18. Skeletal abnormalities: Comment on radiological abnormalities. If bone age was done please note. Comment on scoliosis.
19. Central nervous system: Document if any imaging done and what was found. Careful documentation of neurological exam, especially regarding tone. Ask regarding seizures.
20. Developmental milestones: Record in months/weeks
21. Development: rough estimation using guidelines
22. Investigations done: please note when done
23. Tumour surveillance: Note intervals of surveillance
24. Tumours found: Note carefully the findings, management and outcome

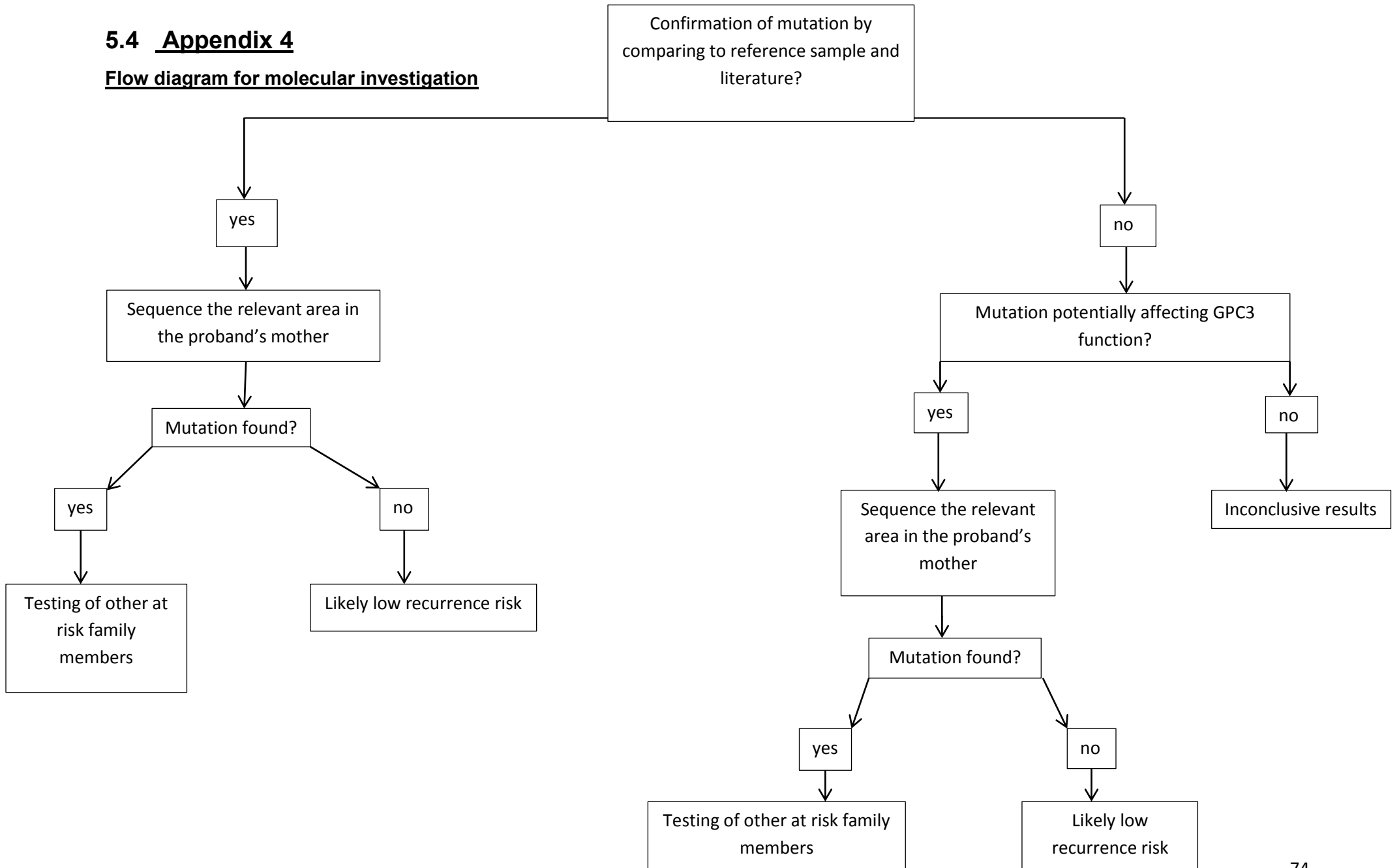
5.3 Appendix 3

Flow diagram for molecular investigation



5.4 Appendix 4

Flow diagram for molecular investigation



5.5 Appendix 5: English version

UNIVERSITY OF CAPE TOWN

DIVISION OF HUMAN GENETICS

INFORMED CONSENT FOR RESEARCH PROJECT:

A clinical and molecular investigation of Simpson-Golabi-Behmel syndrome

I,, hereby voluntarily consent to my child / my genetic material being investigated for a disease causing mutation in the potential genes causing Simpson-Golabi-Behmel syndrome(SGBS) in my family.

INFORMATION REGARDING RESEARCH STUDY:

The aims of this study are:

1. To clinically describe family members with, or at risk of being carriers of SGBS
2. To look for a disease causing mutation in potential genes implicated in SGBS
3. If a mutation is found in an affected person, to look for the disease causing mutation in at risk family members

Each participant may have:

1. A clinical examination done by one of the medical doctors at the Division of Human Genetics
2. A blood / saliva sample taken to extract DNA

Initially only DNA of affected persons will be analysed for mutations in genes implicated in SGBS, if any are found the at risk family member's DNA will be tested 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 SGBS.

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 data.

No identifiable data will be used in publication.

Photos may be published with your consent.

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

Signature: Date:

Witness signature: Date:

5.6 Appendix 5: Afrikaanse weergawe

UNWERSITEIT VAN KAAPSTAD

AFDELING VAN MENSLIKE GENETIKA

INLIGTE TOESTEMMING VIR NAVORSINGSPROJEK:

'n Kliniese en molekuleêre ondersoek van Simpson-Golabi-Behmel sindroom

Ek, gee hiermee vrywillig toestemming dat my kind /my genetiese materiaal ondersoek mag word vir 'n mutasie in die moontlike gene wat Simpson-Golabi-behmel sindroom (SGBS) in my familie veroorsaak.

INLIGTING AANGAANDE DIE NAVORSINGSPROJEK:

Die doelwitte van hierdie navorsingsprojek is as volg:

1. Om die kliniese beeld van familieledede met SGBS, asook die wat 'n risiko loop om draers van SGBS te wees, te beskryf.
2. Om in die gene wat moontlik SGBS veroorsaak te soek vir 'n mutasie wat verantwoordelik is vir SGBS.
3. Indien 'n mutasie wat SGBS veroorsaak gevind word in 'n geaffekteerde persoon, om in hoë risiko familieledede na die mutasie te soek.

Elke deelnemer aan die studie kan die volgende verwag:

1. 'n Kliniese ondersoek deur een van die dokters by die Afdeling van Menslike Genetika
2. 'n Bloed / spoeg monster om DNA van te isoleer.

Aanvanklik sal net die DNA van geaffekteerde seuns ondersoek word vir mutasies in die gene wat SGBS kan veroorsaak.

Indien 'n mutasie gevind word sal hoë risiko familieledede se DNA ondersoek word.

Deur u toestemming tot deelname aan die studie te gee, gee u ook toestemming dat u DNA gestoor mag word vir toekomstige gebruik. Enige verdere genetiese toetsing sal net oor SGBS handel.

Alle uitslae sal aan die einde van die studie aan die relevante persone bekend gemaak word, waar moontlik. Gevolge van hierdie uitslae sal in detail bespreek word tydens 'n genetiese beradingsessie.

Toestemming tot deelname in die studie sluit ook toestemming tot publikasie in.

Geen identifiseerbare data sal gebruik word nie.

Foto's mag gepubliseer word met u toestemming.

Deelname aan hierdie studie is vrywillig, en onttrekking kan op enige stadium geskied. Indien u onttrek sal daar geen negatiewe uitwerking op u behandeling of sorg wees nie.

Handtekening: Datum:

Getuie handtekening Datum:

5.7 Appendix 6 : English Version

INFORMATION SHEET FOR PARTICIPANTS OF RESEARCH STUDY:

A clinical and molecular investigation of Simpson-Golabi-Behmel syndrome

1. You have been approached to participate in this study because of your or your family's history of Simpson-Golabi-Behmel syndrome (SGBS).
2. People with SGBS are often big babies when they are born; they also tend to be taller adults than others. People with SGBS also share similar facial features. Some people with SGBS have other problems too, such as with their hearts, backs, or kidneys. People with SGBS may also have difficulties with learning. The other important thing is that people with SGBS could have some growths in their kidneys and livers, and they need regular monitoring for these.
SGBS is an X-linked disorder; this means that usually it is boys who are affected with it, although sometimes girls could also have a few signs of it. Usually however, women are only "carriers" of the genetic change causing SGBS. If a woman is a carrier of this change, she probably has no problems herself, but will have a 50% chance of passing it on to her son – causing him to have SGBS.
3. This study will look for a change the *GPC3* gene in boys with SGBS.
4. If a change is found, further investigation into at risk family members will be done. If no change is found, family members' blood will not be investigated for the change.
5. As part of this research, a clinical examination may be done on participants by one of the doctors working in the Division of Human Genetics of the University of Cape Town. Secondly a sample of blood/saliva may also be taken to isolate your genetic material.
6. The risk to you during the peripheral blood taking is the same as with routine blood taking. Should we gather a saliva sample from you there will be no physical risk involved.
7. Your genetic material will be stored safely, and will not be used for any other purposes, other than SGBS.
8. All the information gathered during this study will be safely stored in locked offices or in password protected computers.
9. When this information is published it will be done without any identifiable data.
10. Photographs may be published with your consent, but the eyes will be covered.

11. If your genetic material was analysed for the change seen in SGBS the result will be given to you in person (where possible), and explained in detail. All effort will be made to contact you. Please also inform us if your contact details change during the study period.
12. If at any time during the study you wish to withdraw your consent you may do so. There will be no penalties to your future care if you decide to withdraw from the study.
13. Please feel free to contact the following people if you have any problems or questions about the research:

Dr Careni Pretorius

Tel: 021 406 6698

E-mail: ce.pretorius@uct.ac.za

Dr Karen Fieggen

Tel: 021 406 6298

E-mail: karen.fieggen@uct.ac.za

Sr Legg/ Sr Sklar

Tel: 021 406 6304

14. 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.

5.8 Appendix 6 : Afrikaanse weergawe

INLIGTINGSTUK VIR DEELNEMERS AAN NAVORSINGSPROJEK:

‘n Kliniese en Molekulêre ondersoek van Simpson-Golabi-Behmel sindroom

1. U is genader om aan die navorsingsprojek deel te neem as gevolg van u of u familie se geskiedenis van Simpson-Golabi-Behmel sindroom (SGBS).
2. Mense met SGBS is gewoonlik groot babas met geboorte, hulle is ook meesal langer as ander volwassenes. Somige mense met SGBS het ander probleme ook, soos met hulle hart, niere, of ruê. Mense met SGBS het ook soms leerprobleme. Die ander belangrike ding is dat mense met SGBS groeisels in hulle lewers en niere kan kry, daarom benodig hulle gereelde ondersoek daarvoor.
SGBS is ‘n X-gekoppelde sindroom, dit beteken dat seuns meesal geaffekteer word, terwyl vroue net “draers” is van die genetiese verandering. Somtyds kan vroue egter ‘n paar tekens van SGBS hê. Indien ‘n vrou ‘n draer is van die genetiese verandering, het sy ‘n 50% kans om dit aan haar seun oor te dra – hy sal dan SGBS hê.
3. Hierdie navorsingsprojek gaan vir ‘n verandering in die *GPC3* geen soek in seuns met SGBS. Veranderinge in die geen SGBS kan veroorsaak.
4. Indien ‘n verandering wat ons dink verantwoordelik is vir SGBS gevind word in ‘n seun met SGBS, sal ons verdere ondersoek instel na verdere familieledede. Indien ons geen verandering vind in ‘n seun met SGBS, sal ons nie verder na ander familieledede se genetiese materiaal kyk nie.
5. As deel van hierdie navorsing mag deelnemers ‘n kliniese ondersoek deur een van die dokters van die Afdeling van Menslike Genetika ondergaan. Verder mag daar ook ‘n bloed of spoeg monster geneem word om genetiese materiaal te kry.
6. Die risiko’s verbonde aan die bloed monster is dieselfde as met ‘n roetine blootrekking. Indien u ‘n spoeg monster verskaf is daar geen fisiese risiko tot u nie.
7. U genetiese materiaal sal veilig gestoor word, dit sal nie vir ander doeleindes buiten SGBS gebruik word nie.
8. Al die inligting wat tydens die navorsing ingewin word sal in ‘n geslote kantoor, of in ‘n rekenaar met ‘n sleutelwoord gestoor word.
9. Wanneer die navorsing gepubliseer word, sal dit gedoen word sonder identifiseerbare inligting
10. Foto’s mag gepubliseer word met u toestemming, mits die oë weggesteek word.

11. Indien u genetiese materiaal geanaliseer is vir die verandering in SGBS, sal die uitslag persoonlik (waar moontlik) vir u gegee word. Dit sal ook in meer detail bespreek word. Alle voorsiening sal gemaak word om u in die hande te kry, maar indien moontlik lig ons asseblief in indien u kontakinligting verander.
12. Indien u op enige stadium tydens die navorsing u toestemming wil onttrek mag u so doen. Daar sal geen negatiewe uitwerking op u behandeling of sorg wees nie.
13. Kontak enige van die volgende mense gerus indien u enige probleme met of vrae oor die navorsing het:

Dr Careni Pretorius

Tel: 021 406 6698

E-pos: ce.pretorius@uct.ac.za

Dr Karen Fieggen

Tel: 021 406 6298

E-mail: karen.fieggen@uct.ac.za

Sr Legg/Sr Sklar

Tel: 021 406 6304

14. As enige vrae oor 'n regte as deelnemer aan hierdie navorsingsprojek het, kontak gerus Prof M Blockman, die Voorsitter van die Etiese Hersiening Komitee van die Universiteit van Kaapstad by 021 406 6496.

5.9 Appendix 7: English Version

UNIVERSITY OF CAPE TOWN

DIVISION OF HUMAN GENETICS

ASSENT FOR RESEARCH PROJECT:

A clinical and molecular investigation of Simpson-Golabi-Behmel syndrome

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 Simpson-Golabi-Behmel syndrome (SGBS)
2. You and some members of your family have been asked to participate in the study because SGBS is in your family.
3. The doctors are trying to see if they can learn more about how someone with SGBS looks, and how it happens. This is so they can better help your 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 saliva (spit) from you. The way they collect the saliva is by asking you to spit into a container.
5. Nothing that will be done will be painful, or 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 what you did.
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 it 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:

5.10 Appendix 7: Afrikaanse weergawe

UNWERSITEIT VAN KAAPSTAD

AFDELING VAN MENSlike GENETIKA

INSTEMMINGSVORM VIR NAVORSINGSPROJEK

'n Kliniese en molekuleêre ondersoek van Simpson-Golabi-Behmel sindroom

1. Die dokters by die Universiteit van Kaapstad is besig om 'n studie te doen oor 'n genetiese toestand wat mens Simpson-Golabi-Behmel sindroom (SGBS) noem.
2. Jy en van jou familie is uitgenooi om aan die studie deel te neem omdat SGBS in jou familie is.
3. Die dokters doen hierdie studie om te sien of hulle meer kan leer van hoe iemand met SGBS lyk, en ook hoe dit gebeur. Dit is sodat hulle jou, en ander families met SGBS in die toekoms nog beter kan help.
4. As jy besluit om aan die studie deel te neem, sal een van die dokters van Genetika jou ondersoek, en ook 'n spoeg monster neem van jou. Hulle sal hierdie spoeg versamel deur vir jou te vra om in 'n buisie te spoeg.
5. Niks wat in die studie gedoen word sal seer wees, of jou in gevaar stel nie.
6. Alles wat tydens die studie gedoen word sal geheim gehou word, en niemand wat nie 'n deel was van die studie sal weet wat jy gedoen het nie.
7. Jy mag self besluit of jy will deel wees van hierdie studie. As jy besluit om nie deel te neem nie sal niemand vir jou kwaad wees nie, en jy sal nogsteeds al die sorg kry wat jy nodig het.

Handtekening: Datum:

Getuie handtekening Datum:

5.11 Appendix 8

Funding for MMED investigating 2 patients with Simpson-Golabi-Behmel syndrome

Introduction:

Simpson-Golabi-Behmel syndrome (SGBS) is an X-linked skeletal overgrowth disorder. Its incidence is not known but is believed to be under diagnosed. The disorder is characterised by macrosomia, distinctive facial features and multiple congenital abnormalities. The birth weight or length is usually above the 95th centile, reflecting prenatal onset of overgrowth. Distinctive facial features include macrocephaly, hypertelorism, epicanthic folds and downslanting palpebral fissures. The facies is coarse, with a broad nasal bridge and anteverted nares. Macrostomia and macroglossia with a grooved tongue are also present. Congenital abnormalities can include a cleft palate, congenital heart lesions, conduction defects, supernumerary nipples and umbilical hernia. Renal and genital abnormalities have been reported, as have skeletal abnormalities leading to lumbar lordosis. Although normal development can occur, a spectrum of intellectual disability ranging from mild to severe is common. Importantly, in SGBS there is about a 10% chance of developing a malignant tumour. The 5 tumour types described in SGBS are Wilms tumour, hepatoblastoma, adrenal neuroblastoma, gonadoblastoma and hepatocellular blastoma. Heterozygous females can have some mild manifestation of SGBS due to skewed X-inactivation.

Mutations in two genes have been associated with SGBS - *GPC3* and *GPC 4*. These genes are adjacent to each other at the Xq26 locus. Sequence analysis of *GPC3* can identify up to 70% of clinically affected males. *GPC3* consists of 8 exons and span 500kb. Numerous different mutations have been reported in all 8 exons of *GPC3*. 50% of *GPC3* mutations are deletions in exon 8. *GPC4* comprises 9 exons, but testing of this gene is only currently available on a research basis. It is uncertain how abnormalities in these genes cause SGBS, it is however known that the normal gene product of *GPC3* is involved in cell growth and division.

Aim of this MMED research project:

Two patients with a clinical diagnosis of SGBS have been seen by the Division of Human Genetics at the University of Cape Town. DNA is available from both these boys, as well as their mothers. The aim of this MMED project is twofold. The first aim is to delineate the history, clinical phenotype and general manifestations of these two patients. The second aim is to sequence the *GPC3* gene in an attempt to find a disease causing mutation. As far as known, testing of *GPC3* gene for SGBS is not routinely available in South Africa.

Outcomes:

SGBS is inherited, and thus if a disease causing mutation is found there will be implications for the family. This project could also result in the availability of diagnostic testing, and later on prenatal testing.

Breakdown of funding needed:

AIM 2: Molecular investigations

Primers, PCR, sequencing of the 8 exons of *GPC3* :
R 16 000.00

Printing, binding and miscellaneous costs:

Printing:
R 600.00

Miscellaneous:
R 400.00

TOTAL (estimated cost)
R 17 000.00
