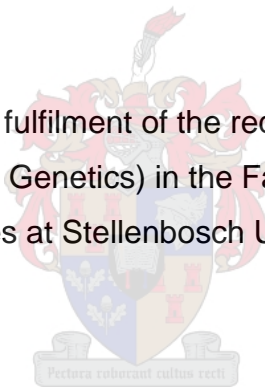


**LETHAL MULTIPLE PTERYGIUM SYNDROME:
A SOUTH AFRICAN CASE SERIES WITH GENOMIC INVESTIGATION
USING WHOLE EXOME SEQUENCING**

Dr Liani Smit

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Medicine (Medical Genetics) in the Faculty of Medicine and Health
Sciences at Stellenbosch University



Supervisor: Prof Michael Urban
Co-supervisor: Dr Caitlin Uren
Date: December 2020

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Liani Smit

Abstract

Introduction:

Lethal multiple pterygium syndrome (LMPS) is a rare and lethal neuromuscular disorder of the fetus. Cases are characterised by absent fetal movement (fetal akinesia) causing arthrogyrosis with pterygia of major joints and inevitable intrauterine lethality. LMPS is a genetically heterogenous single gene disorder, following either autosomal or X-linked recessive patterns of inheritance. Epidemiologic, phenotypic, and genomic LMPS data have not been established in a South African population.

Methods:

Cases matching the LMPS definition were ascertained retrospectively (2011-2015) and prospectively (2016-2018) from medical genetic and fetal medicine records at Tygerberg Hospital. Comprehensive phenotyping was performed using prenatal ultrasound, clinical, photographic, radiologic and autopsy sources. Genomic investigation using whole exome sequencing (WES), was undertaken in an initial trio (affected fetus and unaffected parents) aimed at detecting disease-causing variants in known or novel genes associated with LMPS.

Results:

Over an 8-year period, 20 women with 25 affected fetuses (11 females, 10 males, 4 unknown sex) were identified of predominantly Black South African ancestry (18/20). 20% of women had known LMPS recurrence with the same non-consanguineous partner. Our data support an estimated LMPS prevalence rate of 1 in 20,000 in our referral area which manages approximately 50,000 deliveries per year. LMPS or non-viability was antenatally recognized in 72% (18/25), with 78% (14/18) of women opting for termination of pregnancy. Half of women (2/4) who continued, developed complications, i.e. preeclampsia and hydrops fetalis precluding vaginal delivery (1/4) and severe polyhydramnios with acute severe hypertension (1/4). Antenatal non-recognition of non-viability (7/25) occurred outside the Fetal Medicine unit and often resulted in unnecessary Caesarean section (2/7). First trimester sonography had a 100% (3/3) detection rate of severe fetal akinesia, i.e. multiple fixed flexion joint deformities, increased nuchal translucency, generalised oedema and reduced or

absent fetal movements, but not pterygia. In addition to these findings, 2nd and 3rd trimester fetal anatomy sonography in 16 pregnancies detected abnormal positioning of the feet (75%), pulmonary hypoplasia (63%), micrognathia (56%), pterygia (50%) and camptodactyly (50%). Fetal hydrops increased from 66% during the 1st trimester to 80% after 24 weeks. Dysmorphology assessments (22/25) supplemented by photographic phenotyping (7/25), radiologic (6/25) and autopsy (10/25) examinations supported antenatal findings. Several patterns emerged, including similar facial dysmorphology (5/7), abnormal curvature of the spine (6/6) and evidence for possible cardiac and smooth muscle involvement, i.e. cardiac hypoplasia (2/10) on autopsy, and genitourinary tract dilatation (5/25) on ultrasound. Muscle histology was non-contributory, though immunohistochemistry was unavailable. Initial trio WES did not detect disease-causing variants in known LMPS or fetal akinesia genes but identified *ASCC3* is a possible gene of interest in LMPS.

Conclusion:

Our data suggest a 50-fold increased incidence of LMPS in our population compared to previous international estimates and appears more common among Black South Africans. Dysmorphic, X-ray and autopsy findings are similar to previous case reports, with additional findings suggesting possible cardiac and smooth muscle involvement in addition to skeletal muscle. The genetic basis of LMPS in our population remains uncertain as no causative mutations were detected on a single trio subjected to WES. While genetic heterogeneity is possible, our case series supports an autosomal recessive pattern of inheritance, with recurrence risk implications for couples. Severe fetal akinesia is detectable from 1st trimester ultrasound and the presence of hydrops fetalis should prompt review for evidence of fetal akinesia. Early recognition of LMPS and non-viability allows for improved pregnancy management. In ongoing pregnancies there is a need for awareness of increased risk of pregnancy complications and attention to appropriate delivery management. Further genomic investigations may clarify the genetic contribution of LMPS in our population, which could be unique.

Opsomming

Inleiding:

Letale meervoudige pterygium sindroom (LMPS) is 'n raar en dodelike neuromuskulêre kondisie van die fetus. Gevalle word gekenmerk deur afwesige fetale bewegings (fetale akinesie), met gevolglike veelvuldige kontrakture van die gewrigte (artrogripose) met pterygia en onvermydelike intrauteriene sterfte. Die genetiese oorsake van LMPS is heterogeen en volg 'n outosomaal of X-gekoppeld resessiewe oorerflikheidspatroon. Epidemiologiese, fenotipiese en genomiese data is nie van te vore in die Suid-Afrikaanse populasie vasgestel nie.

Metodes:

Gevalle wat aan die LMPS definisie voldoen is terugwerkend (2011-2015) en voornemens (2016-2018) identifiseer van mediese genetiese en fetale medisyne rekords by Tygerberg Hospitaal. Omvattende fenotipering is uitgevoer vanuit kliniese, fotografiese, radiologiese en nadoodse ondersoek bronne. 'n Genomiese ondersoek deur middel van WES is onderneem in 'n aanvanklike trio (geaffekteerde fetus en beide ongeaffekteerde ouers) met die doel om genetiese variante op te spoor in bekende of nuwe gene geassosieer met LMPS.

Resultate:

Oor 'n 8-jaar periode is 20 vroue met 25 geaffekteerde fetusse (11 vroulik, 10 manlik en 4 onbekende geslag) identifiseer van hoofsaaklik Swart Suid-Afrikaanse afkoms (18/20). 20% van vroue het 'n herhaling van LMPS gehad met dieselfde onverwante maat. Ons data ondersteun 'n beraamde LMPS prevalensie van 1 in 20,000 in ons verwysingsarea wat ongeveer 50,000 bevallings jaarliks behartig. LMPS of nie-lewensvatbaarheid is voorgeboortelik herken in 72% (18/25), met 78% (14/18) van vroue wat terminasie van swangerskap gekies het. Die helfde (2/4) van vroue wie voortgegaan het met die swangerskap het komplikasies ondervind, naamlik preeklampsie en fetale hydrops wat vaginale verlossing belemmer (1/4) en erge polihidramnios met ernstige akute hipertensie (1/4). Gebrek aan voorgeboorte herkenning van nie-lewensvatbaarheid (7/25) buite die Fetal Medisyne eenheid, het dikwels gelei tot onnodige keisersnitte (2/7). 1^{ste} trimester sonografie het tekens van ernstige fetale akinesie suksesvol in 100% van gevalle identifiseer, naamlik

veelvuldige gewrigskontrakte, vergrote NT met algemene edeem en verminderde of afwesige fetale bewegings, maar nie pterygia nie. Bykomend tot hierdie tekens het 2^{de} en 3^{de} trimester fetale anatomie sonografie in 16 swangerskappe abnormale positionering van die voete (75%), long hipoplasie (63%), mikrognatie (56%), pterygia (50%) en kamptodaktilie (50%) getoon. Die teenwoordigheid van fetale hidrops het vermeerder van 66% tydens 1^{ste} trimester sonografie tot 80% na 24-weke gestasie. Dismorfologiese (22/25), supplementele fotografiese fenotipering (7/25), radiologiese (6/25) en nadoodse ondersoeke (10/25) het die bevindinge op voorgeboorte sonografie ondersteun. Verskeie patrone het na vore gekom, insluitend soortgelyke gesigsdismorfologie (5/7), abnormale kurwatuur van die ruggraat (6/6), asook bewyse van moontlike hart- en gladde-spier betrokkenheid, naamlik hart hipoplasie (2/10) op nadoodse ondersoek en dilatasie van die genitourinêre traktus (5/25) op sonar. Spierhistologie was nie bydraend nie, alhoewel immunohistochemie nie geredelike beskikbaar was nie. Aanvanklike trio WES het geen patogene variante getoon in bekende LMPS of fetale akinesie gene nie, maar het *ASCC3* as 'n moontlike geen van belang in LMPS identifiseer.

Samevatting:

Ons data dui op 'n 50-maal verhoogde insidensie van LMPS in ons populasie in vergelyking met vorige internasionale beramings en blyk meer algemeen onder Swart Suid-Afrikaners. Dismorfologiese, X-straal en nadoodse ondersoek bevindinge is soortgelyk aan vorige gevallereekse, met addisionele uitslae wat ook op moontlike hart-en gladde-spier betrokkenheid mag dui. Die genetiese basis van LMPS in ons populasie bly onduidelik in die afwesigheid van enige veroorsakende mutasies na trio WES. Terwyl genetiese heterogeniteit moontlik is, ondersteun ons gevallereeks 'n outosomaal resessiewe patroon van oorwerwing met herhalingsrisiko implikasies vir paartjies. Ernstige fetale akinesie is alreeds waarneembaar tydens 1^{ste} trimester sonografie en die teenwoordigheid van hidrops fetalis noodsaak 'n deeglike ondersoek vir tekens van fetale akinesie. Vroeë herkenning van LMPS en nie-lewensvatbaarheid, bewerkstellig verbeterde hantering van swangerskappe. Bewusmaking rakende verhoogde risiko vir komplikasies en gepaste hantering van bevallings is noodsaaklik waar swangerskappe voortgaan. Verdere genomiese ondersoeke sal moontlik meer duidelikheid rakende die genetiese bydrae tot LMPS in ons populasie bring.

Acknowledgements

I am grateful for the contribution, guidance, and support of the following people throughout the duration of this project:

- My supervisor, **Prof Michael Urban**, for his mentorship, commitment to developing my critical thinking skills, and invaluable constructive critique from conceptualisation of the project to the final draft
- My co-supervisor, **Dr Caitlin Uren**, for helping me navigate the world of genomics and bioinformatic analyses
- The Tygerberg Fetal Medicine unit and prenatal ultrasound team, especially **Prof Lut Geerts** and **Dr Kerry Rademan**, for their meticulous ultrasound assessments that were integral to the prenatal identification and description of these cases
- My past and present colleagues in the **Medical Genetics team** for their patience, encouragement and contribution to the identification and counselling of families affected by LMPS
- The **postgraduate students** in the MAGIC laboratory for the 'wet' lab work
- Tygerberg Anatomical Pathology, especially **Dr Pawel Schubert**, and the 1st floor radiography team
- A special thank you to the **families affected by LMPS** who agreed to participate in this project which we hope will improve our understanding of an understudied and probably underrecognized condition in South Africa
- Finally, my mom **Elisabet**, who taught me that with hard work and a few sacrifices I could achieve anything I aspire to

Table of Contents

Declaration.....	ii
Abstract.....	ii
Opsomming	iv
Acknowledgements.....	vi
List of Tables	ix
List of Figures	x
List of Abbreviations.....	xi
Glossary.....	xiv
1. Introduction.....	1
1.1 Background.....	1
1.2 Problem Statement.....	2
1.3 Purpose of the study.....	2
1.4 Aims.....	3
1.5 Objectives	3
2. Literature Review	4
3. Research Methodology	15
3.1 Study Setting.....	15
3.2 Study Population	15
3.3 Study Design.....	16
3.3.1 Descriptive case series.....	16
3.3.2 Genomic investigation and analysis using WES and bioinformatics.....	17
3.4 Data Collection.....	19
3.4.1 Clinical Data collection	19
3.4.2 Genomic investigation using WES.....	21
3.5 Data Analysis	22
3.5.1 Clinical data analysis.....	22
3.5.2 Genomic data analysis and the bioinformatics pipeline	22
3.5.2.1 The bioinformatics pipeline	22

3.5.2.2	Variant annotation and filtering using standardised tools	23
3.5.2.3	Variant interpretation and classification.....	24
3.5.2.4	Incidental findings	26
3.6	Ethics approval and other ethical considerations.....	26
3.6.1	Data management and privacy.....	27
3.6.2	Genetic counselling and informed consent for genomic investigation	27
3.6.3	Storage of genomic data	28
3.6.4	Risk to study participants: blood sample collection	28
3.6.5	Benefits to study participants.....	28
4.	Results: Descriptive Case series.....	29
4.1	Results overview	29
4.2	Demographic information.....	29
4.3	Maternal health.....	31
4.4	Pregnancy outcomes and complications.....	31
4.5	Recurrence of LMPS	35
4.6	Prenatal ultrasound findings	35
4.7	Phenotypic features on external examination of the fetus	41
4.8	Macroscopic and microscopic findings on autopsy	47
4.9	Radiographic features.....	47
4.10	Placental findings	52
4.11	Results of standard of care genetic investigations	52
5.	Results: Genomic investigation using WES and bioinformatics	53
5.1	Variant annotation and filtering	53
5.2	Identification, interpretation, and classification of variants in genes of interest.....	55
5.3	Further variant interpretation and classification of candidate variant(s).....	58
5.4	Incidental findings.....	60
6.	Discussion	61
7.	Conclusion and future research directions	75
8.	References	78
9.	Appendix A: Perinatal Losses Data Sheet.....	84

List of Tables

Table 1	Number of LMPS cases per year with sex distribution	29
Table 2	Overview of women and affected fetuses	30
Table 3	Pregnancy outcomes with mean gestational age	32
Table 4	First trimester NT ultrasound findings	36
Table 5	Second and third trimester ultrasound findings	37-38
Table 6	Findings on ultrasounds performed outside the Fetal Medicine unit	40
Table 7	Phenotypic features and dysmorphology on external fetal examination	42-43
Table 8	Macroscopic and microscopic findings on autopsy	48
Table 9	Radiographic features on postmortem fetogram	49
Table 10	Taper™ variant filtering in the proband	54
Table 11	Varseq® variant filtering in the proband	54
Table 12	Candidate variants interpretation and classification	56
Table 13	ACMG/AMP variant classification for <i>ASCC3</i> c.299C>T	60

List of Figures

Figure 1	The relationship between primary causes of fetal akinesia and secondary fetal effects	8
Figure 2	The seven-level filtration framework and backbone of TAPER™	24
Figure 3	Evidence framework for assigning pathogenicity (ACMG-AMP guidelines, 2015)	25
Figure 4	The relationship between prenatal detection of LMPS or fetal non-viability with pregnancy outcomes, mode of delivery and associated complications	33
Figure 5	Frontal photographs	44
Figure 6	Lateral photographs	44
Figure 7	Posterior photographs	45
Figure 8	Musculoskeletal anomalies of the upper and lower limbs.	45
Figure 9	External features of a 12 weeks fetus	46
Figure 10	Anteroposterior fetograms	50
Figure 11	Lateral fetograms	51
Figure 12	The ASC-1 pathway with <i>ASCC3</i> and interactor genes, including <i>ASCC1</i> and <i>TRIP4</i>	57

List of Abbreviations

1KGP	1000 Genomes Project
ACHR	Acetylcholine receptor
ACMG	American College of Medical Genetics
AGA	Average for gestational age
AMC	Arthrogryposis multiplex congenita
AMP	Association for Molecular Pathology
ANNOVAR	Annotate Variation
AD	Autosomal dominant
AR	Autosomal recessive
ASC-1	Activating signal co-integrator 1
ASHPT	Acute severe hypertension
B	Black African
Br	Breech
BWA	Burrows-Wheeler Aligner
C	Cephalic
CADD	Combined Annotation Dependant Depletion
CHPT	Chronic hypertension
CNV	Copy number variation or copy number variant
CNS	Central nervous system
CS	Caesarean section
DCDA	Dichorionic diamniotic
DNA	Deoxyribonucleic acid
DV	Ductus venosus
EF	Echogenic focus
ENND	Early neonatal death
ESP6500	Exome Sequencing Project 6500
EVMP5	Escobar variant multiple pterygium syndrome
F	Female
FADS	Fetal akinesia deformation sequence
FATHMM	Functional Annotation Through Hidden Markov Models
FC	Feticide
GATK	Genomic Analysis Toolkit

GERP	Genomic Evolutionary Rate Prediction Score
GI	Gastrointestinal
GNOMAD	Genome Aggregation Database
HET	Heterozygous
HIV	Human Immunodeficiency Virus
HMZ	Homozygous
HN	Hydronephrosis
HPO	Human Phenotype Ontology
HREC	Health Research Ethics Committee
IGV	Integrative Genomics Viewer
INDEL	Insertion deletion polymorphism
IUD	Intrauterine death
IUGR	Intrauterine growth restriction
LGA	Large for gestational age
LMPS	Lethal multiple pterygium syndrome
M	Male
MAF	Minor allele frequency
MD	Mendelian disorder
MDE	Major depressive episode
MTOP	Medical termination of pregnancy
N	Normal
NA	Not applicable
NGS	Next generation sequencing
NF	Nuchal fold
NR	Not reported
NT	Nuchal translucency
OMIM	Online Mendelian Inheritance of Man
PE	Preeclampsia
PIIP	Perinatal Problem Identification Programme
PPROM	Preterm prelabour rupture of membranes
PT	Prenasal thickness
PTL	Preterm labour
QFPCR	Quantitative Fluorescent Polymerase Chain Reaction
RPOC	Retained products of conception

S	Singleton
SGA	Small for gestational age
SIFT	Sorting Intolerant From Tolerant
SNV	Single nucleotide variant
STOP	Surgical termination of pregnancy
SUA	Single umbilical artery
SU	Stellenbosch University
T	Trimester
TOP	Termination of pregnancy
U	Unknown
V	Vaginal
VCF	Variant Call Format
VUS	Variant of uncertain significance
VM	Ventriculomegaly
WES	Whole exome sequencing
WGS	Whole genome sequencing

Glossary

Akinesia	Absence of muscle movement
Allele	Alternative forms of a gene
ANNOVAR	A variant annotation tool
Aplasia	Failure of normal organ or tissue development
Apgar score	Standardised scoring tool for assessment of a new-born immediately after birth
Arthrogryposis	Congenital joint contractures in two or more areas of the body
Atrophy	Decrease in size or wasting away of a body part or tissue
Biallelic	Pertaining to both alleles
Bioinformatics	The collection, classification, storage, and analysis of biochemical and biological information using computers especially as applied to molecular genetics and genomics
CADD score	Computational scoring tool for predicted deleteriousness of SNVs and indels by integrating multiple annotations including conservation and functional information into one metric
Cis	On the same side
ClinVar	Freely accessible and public archive of reports of the relationships among human variations and phenotypes with supporting evidence
Compound het	Two different mutant alleles at a gene locus, one on each chromosome
CNV	DNA segment of one kilobase or larger that is present at a variable number in comparison with a reference genome
Cystic hygroma	Cyst(s) caused by an underlying abnormality of lymphatic development, usually located in the neck
Deformation	Alteration in the shape or structure of an organ or tissue
Dysmorphology	Study of congenital structural malformations or anomalies
Epigenetics	Study of heritable changes in gene expression
FATHMM	Computational tool which predicts the functional consequence of coding and non-coding variants
Feticide	Procedure resulting in cessation of fetal cardiac activity prior to the commencing of the termination of pregnancy procedure

Fetogram	X-ray of the entire body of a fetus
Founder effect	Loss of genetic diversity when a small population is separated from a larger gene pool
Frameshift variant	Insertion or deletion involving several DNA base pairs that is not a multiple of three, which disrupts the triplet reading frame
Genetic drift	Change in allele frequency in a population due to a random selection of certain genes
Genotype	Genetic constitution of an individual
GERP score	Conservation score calculated by quantifying substitution deficits across multiple alignments of orthologues using the genomes of 35 mammals
GnomAD	Collection of exome and genome sequencing data sets from unrelated individuals sequenced as part of various disease specific or population genetic studies
Heterozygous	Different alleles at corresponding loci on homologous chromosomes
Homozygous	Identical alleles at corresponding loci on homologous chromosomes
Hydrops fetalis	Abnormal accumulation of fluid in two or more fetal compartments
Hypokinesia	Reduced muscle movement
Hypoplasia	Underdevelopment or incomplete development of a tissue or organ
Hypoxia	Deficiency in amount of oxygen reaching tissues
Ischaemia	Deficiency in blood supply to tissues
Large for GA	Birth weight above 90 th percentile for gestational age
Megacystis	Abnormally large or distended bladder
MAF	The frequency at which the second most common allele occurs in a population
Micrognathia	Reduced heights and width of the mandible when viewed from the front
Missense variant	Single base pair change resulting in a different amino acid
Mirror syndrome	Co-occurrence of both maternal hypertension and oedema with either fetal and/or placental hydrops

Miscarriage	Spontaneous abortion of a fetus before 20 weeks of pregnancy
NGS	Massively parallel and high-throughput DNA sequencing
Nonsense variant	Substitution of a single base pair that leads to a premature stop codon
Gene ontology	Major bioinformatics initiative to unify the representation of gene and gene product attributes across all species
Phenotype	The set of observable characteristics of an individual
Polymorphism	Variant present in >1% of the population
Pterygia	Webbing of the skin over joints
Sanger sequencing	Method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleosides by DNA polymerase
Sequencing	Process of determining the nucleic acid sequence
SIFT	Computational tool which predicts the effect of amino acid substitution on protein function based on sequence homology and the physical properties of amino acids
Small for GA	Birth weight below 10 th percentile for gestational age
SNV	Substitution of a single nucleotide for another
Splice-site variant	Genetic alteration in the DNA sequence that occurs at the boundary of an exon and an intron
Stillbirth	A baby born with no signs of life at or after 28 weeks' gestation
STRINGdb	Database of known and predicted protein-protein interactions
Quad	Proband and three additional family members
TOP	Process of ending a pregnancy
Trans	on the opposite side
Trio	Proband and two additional family members

1. Introduction

1.1 Background

Reduced or absent fetal movement in utero (fetal akinesia) results in a clinically and genetically heterogeneous group of disorders. Lethal Multiple Pterygium syndrome (LMPS) exists on the severe end of the fetal akinesia spectrum, resulting in prenatal lethality, usually during the 2nd trimester of pregnancy.

LMPS cases are characterised by the presence of multiple congenital contractures (arthrogryposis) with skin webbing (pterygia) over major joints, and are often associated with hydrops fetalis, pulmonary hypoplasia and intrauterine growth restriction.

LMPS is rare, though exact prevalence rates are largely unknown. LMPS is a single gene (Mendelian) disorder which follows either an autosomal recessive or an X-linked recessive pattern of inheritance. Both patterns of inheritance carry significant recurrence risk implications within families.

High-throughput next generation sequencing (NGS) technology, such as whole exome sequencing (WES) and whole genome sequencing (WGS), enables large scale genomic investigation and diagnosis of genetically heterogeneous Mendelian disorders (MD). Internationally, the application of NGS technologies within the group of fetal akinesia spectrum disorders has resulted in a surge of novel gene and disease-causing variant discovery. More than 320 genes are currently associated with this group of disorders. Genes encoding components of the neuromuscular junction, particularly subunits of the fetal acetylcholine receptor (AChR), and skeletal muscle proteins have been linked to LMPS.

Despite recent international advances towards improved understanding of the pathogenesis and genetics of the fetal akinesia spectrum, little is known about these disorders, especially LMPS, in the South African population.

1.2 Problem Statement

LMPS prevalence rates, phenotypic and genomic data have not been reported in the South African population. Two to three cases of LMPS are diagnosed by our genetics group annually. This prevalence rate appears much higher than those reported internationally. It is unknown whether the epidemiology, phenotype, and genetics of LMPS may be unique in our population.

Prenatal lethal conditions, like LMPS, may go unrecognised and be underdiagnosed in populations with limited access to antenatal care and lack of experience in prenatal ultrasonography. Due to recurrence risk implications, accurate prenatal detection and diagnosis may influence family planning and management in future pregnancies. In addition to clinical diagnosis, making a definitive molecular genetic diagnosis could improve our understanding of the underlying disease aetiology and pathogenesis and provide targets for potential future gene therapies.

Genomic investigation of rare and genetically heterogeneous conditions is challenging since traditional targeted genetic approaches are laborious and largely ineffective. New genomic technologies such as targeted NGS panels, WES and WGS, allow high throughput and large-scale sequencing making this the most appropriate and cost-effective testing approach in genetically heterogeneous conditions.

The cost of NGS technologies have dramatically reduced in recent years yet remains prohibitive for wide-spread application in resource limited settings like South Africa. The amount of data generated with NGS technologies require considerable bioinformatics processing and variant interpretation by a skilled and experienced multidisciplinary team.

1.3 Purpose of the study

The purpose of this case series is to describe the phenotype of LMPS in a South African population and to explore the underlying genetic determinants of LMPS in this

population by conducting an initial genomic investigation using next generation sequencing technology and bioinformatic analysis.

1.4 Aims

The study aims to:

- comprehensively describe the phenotype of LMPS in a South African population presenting to Tygerberg Hospital
- identify potential LMPS disease-causing variants in known or novel genes using whole exome sequencing and various bioinformatic tools

1.5 Objectives

1. Identify cases meeting the LMPS definition in a South African population presenting to Tygerberg Hospital over an 8-year period (2011-2018).
2. Comprehensively describe the phenotype of LMPS in this case series by reporting the following:
 - 2.1. Prenatal ultrasound findings
 - 2.2. Clinical and dysmorphic features on external examination of the fetus
 - 2.3. Macroscopic and microscopic findings on autopsy
 - 2.4. Radiographic features
 - 2.5. Results of standard of care genetic investigations
 - 2.6. Placental features
3. Perform a genomic investigation of LMPS using an NGS platform, i.e. WES
4. Interpret generated sequencing data, using a bioinformatic pipeline, various bioinformatics tools and variant classification systems to establish a list of candidate disease-causing variants.
5. Describe further steps necessary to confirm pathogenicity of novel variants.

2. Literature Review

2.1 Introduction and definitions

Fetal akinesia encompasses a spectrum of clinically and genetically heterogeneous conditions characterized by reduced or absent fetal movement (Ravenscroft *et al.*, 2011; Beecroft *et al.*, 2018; Pergande *et al.*, 2020). Regular fetal movements begin from 7 weeks gestation and are integral to normal development of muscle, bone, joints, lung capacity and gastrointestinal (GI) motility (Lüchinger *et al.*, 2008). Depending on the underlying aetiology and timing of impaired fetal movement, secondary fetal deformation ranges in severity from isolated joint contractures (arthrogryposis) with a reasonable long term quality of life to severe and often lethal types, e.g. fetal akinesia deformation sequence (FADS) and LMPS (Ravenscroft *et al.*, 2011; Hall, 2014; Hall and Kiefer, 2016).

Arthrogryposis refers to joint contractures with limitation of movement in two or more areas of the body that are present at birth (Hall, 2014; Hall and Kiefer, 2016). The term 'arthrogryposis multiplex congenita' (AMC) or 'multiple congenital contractures' are often used interchangeably with arthrogryposis (Hall, 2014; Hall and Kiefer, 2016). Arthrogryposis and AMC do not include isolated clubbed feet or congenital hip dislocation (Hall, 2014). Use and understanding of these various terminologies have evolved over time due to an improved understanding of the pathogenesis and aetiologic heterogeneity, either extrinsic (extra-fetal) or intrinsic (fetal), underlying arthrogryposis. (Hall, 2014). It is now apparent that arthrogryposis and AMC are not specific diagnoses but rather descriptive terms or signs referring to the presence of multiple joint contractures at birth as a result of reduced fetal movement in utero (Hall, 2014; Hall and Kiefer, 2016; Hall, Kimber and Dieterich, 2019).

In the severest form, AMC related to severe and early onset (usually intrinsic) fetal akinesia, result in a characteristic pattern of fetal deformation, recognizable craniofacial dysmorphism and a poor to lethal outcome (Moerman and Fryns, 1990). This pattern was originally recognized by Pena and Shokeir and eponymously named Classic Pena-Shokeir syndrome (Type 1) (Hall, 2009; Nayak *et al.*, 2014). Since these

original reports, an expanded Pena-Shokeir phenotype encompassing different subtypes with distinct underlying aetiologies and neuropathology but with the unifying feature of underlying fetal akinesia is recognized (Moerman and Fryns, 1990; Hall, 2009). The terms FADS and Pena-Shokeir phenotype are now used synonymously (Hall, 2009).

LMPS is considered a phenotypically distinct expression of severe and very early onset fetal akinesia (Hall, 1984; Cox *et al.*, 2003). It shares phenotypic overlap with FADS, e.g. characteristic craniofacial dysmorphism, intrauterine growth restriction (IUGR), and pulmonary hypoplasia, but the presence of fetal hydrops or cystic hygromas with more severe facial dysmorphism, joint contractures and pterygia, distinguishes LMPS from FADS (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990; Moerman and Fryns, 1990).

Several fetal akinesia phenotypes are recognized by the presence of pterygia. Pterygia are webs of skin and soft tissue overlying major joints and are thought to result from reduced mechanical forces on the skin due to reduced fetal movement (Moerman and Fryns, 1990). The presence of pterygia are mandatory in LMPS, whereas only 20% of fetuses with FADS have pterygia (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990). Pterygia may be generalized, involving all or most joints, e.g. in LMPS or non-lethal Escobar variant multiple pterygium syndrome (EVMPS), or be limited to certain joints, e.g. the lower limbs in popliteal pterygium syndrome and Bartsocas-Papas syndrome (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990; Moerman *et al.*, 1990; Parashar *et al.*, 2006). Intrauterine lethality distinguishes LMPS from non-lethal EVMPS (Hall *et al.*, 2018).

2.2 Prevalence of fetal akinesia and LMPS

Although each cause of fetal akinesia is individually rare, arthrogryposis occurs in 1/3000 to 1/5000 live births (Lowry *et al.*, 2010; Hall, 2014). No epidemiological data from populations in Africa have been published.

The incidence of more severe and lethal forms depends on the underlying cause and population being studied. Unique causes of lethal fetal akinesia due to genetic drift or founder effects have been identified in several populations e.g. the Finnish lethal congenital contractual syndrome (Pakkasjärvi *et al.*, 2006) and a Dutch population with FADS (Tan-Sindhunata *et al.*, 2015).

The latest 2020 *Orphanet* report on the prevalence and incidence of rare diseases, reports a European birth prevalence of 5.7/100 000 and 0.6/100 000 for AMC and more severe FADS, respectively (Orphanet, 2020). Due to a limited number of published LMPS case reports, i.e. 28 families with autosomal recessive and 6 families with X-linked recessive LMPS, incidence and prevalence rates have not been established and often quoted as likely less than 1 in 1 million (Orphanet, 2020).

2.3 Aetiology of fetal akinesia and LMPS

Arthrogryposis may result from either extrinsic (extra-fetal) or intrinsic (fetal) causes (Hall, 2014; Hall, Kimber and Dieterich, 2019). Extrinsic causes include limitation of fetal movement due to intrauterine constraint (Hall, 2014), or environmental causes, e.g. vascular compromise of the fetus or placenta (Hall, 2009, 2014), maternal illness, or exposure to infections or teratogens (Hall, 2014). Intrinsic causes are often genetic and usually present with a more severe phenotype, as seen with FADS or LMPS (Hall, 2014).

Intrauterine constraint due to structural uterine anomalies, multifetal pregnancies, oligo- or anhydramnios, or amniotic bands, causes limitation of fetal movement leading to fetal contractures usually from the second half of pregnancy (Hall, 2014). Vascular compromise predisposes the developing fetus to hypoxia and tissue ischemia which may result in either missed developmental steps or damage to vulnerable fetal tissues, including neurons and muscles (Hall, 2014). The cause of fetal vascular compromise often remains unknown, but may include maternal hypotension, significant trauma, monozygotic twinning, or the use of vasoactive medications or drugs during pregnancy e.g. misoprostol or cocaine (Hall, 2009, 2014).

The pathogenesis of fetal akinesia associated with maternal illness, e.g. myasthenia gravis, myotonic dystrophy, diabetes mellitus, and multiple sclerosis, often remains less clear (Hall, 2014). Acquired maternal antibodies against paternally inherited fetal neurotransmitter receptors is currently the only maternal cause easily amenable to therapy during pregnancy (Hall, 2014). Congenital contractures as result of maternal hyperthermia or infections, e.g. rubella or coxsackie, and exposure to medications, e.g. muscle relaxants or antiepileptics (phenobarbitone), have also been reported (Hall, 2014).

Common intrinsic causes of fetal akinesia include: primary defects in the motor system pathway, i.e. the central or peripheral nervous system (major CNS structural malformations, defects of anterior horn cell formation or maintenance and neuropathies), components of the neuromuscular junction, skeletal muscle (congenital dystrophies, myopathies, myositis), or connective tissues (restrictive dermopathies or skeletal dysplasias) (Ravenscroft *et al.*, 2011; Hall, 2014; Beecroft *et al.*, 2018). Metabolic and epigenetic causes of fetal akinesia are also increasingly being recognized (Ravenscroft *et al.*, 2011; Hall, 2014; Beecroft *et al.*, 2018). Over 400 genetic disorders are currently associated with fetal akinesia, including chromosomal abnormalities (e.g. trisomy 18), copy number variations (CNV) and more than 320 single gene disorders (Hall, 2014; Kiefer and Hall, 2019). As observed with amyoplasia, the most common cause of AMC, the cause of fetal akinesia often remains unknown and is likely multifactorial (Hall, 2014).

2.4 Secondary effects of fetal akinesia on fetal development

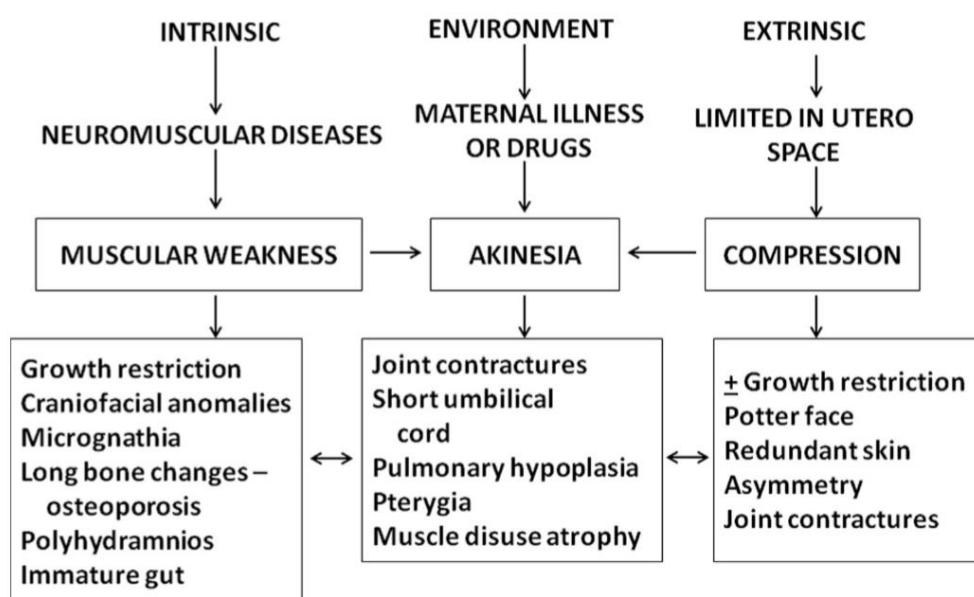
During the 1960's, several studies in animal models demonstrated the importance of fetal movement in normal development (Drachman and Sokoloff, 1966; DeMyer and Baird, 1969). Early fetal immobilization reproduces a recognizable sequence of secondary fetal deformations now known as FADS or Pena-Shokeir phenotype, i.e. arthrogyrosis multiplex congenita with characteristic craniofacial dysmorphism, intrauterine growth restriction (IUGR), pulmonary hypoplasia, shortened GI tract with decreased motility and a shortened umbilical cord (Moessinger, 1983; Hall, 2009). Typical craniofacial dysmorphism associated with FADS includes ocular

hypertelorism, a high nasal root with an underdeveloped tip of the nose, low-set and posteriorly rotated ears, small mouth with limited jaw opening, micrognathia, high-arched or cleft palate, and a short neck (Moessinger, 1983; Hall, 2009).

Decreased fetal movements increase connective tissue surrounding joints, cause disuse muscle atrophy and abnormally shaped joint surfaces, all of which further impede joint mobilization and increase contractures (Swinyard, 1982; Hall, 2014). Therefore, earlier and longer periods of fetal akinesia result in more severe contractures (Hall, 2014). Additional clinical features, e.g. the presence of pterygia, provide further clues as to the timing of fetal akinesia. Following limb and joint formation during the first 8 weeks of gestation, almost a complete lack of movement in the first trimester is required for pterygia to develop (Hall, 2014). Lack of fetal limb movement may result in disuse osteoporosis, predisposing to long bones fractures in the perinatal period (Hall, 2014).

The relationship between the primary cause of fetal akinesia and secondary fetal effects were summarized by Hall in 2014 represented in **Figure 1** (Hall, 2014).

Figure 1: The relationship between primary causes of fetal akinesia and secondary fetal effects (Hall, 2014).



after Thomas & Smith [1974]; Moessinger [1983]; Rodriguez & Palacios [1991]; Hall [2014]

2.5 Expanding the phenotype and pathogenesis of LMPS

Multiple joint contractures, pterygia and intrauterine lethality are characteristic features observed in all fetuses with LMPS (Hall, 1984; Froster *et al.*, 1997; Cox *et al.*, 2003). Hydrops fetalis, cystic hygroma, pulmonary hypoplasia, IUGR and cleft palate are the most common associated findings (Hall, 1984; Froster *et al.*, 1997). Other clinical features are often inconsistently reported leading to a recommendation by Froster *et al.* to follow a consistent pathological workup during the assessment of fetuses with suspected LMPS (Froster *et al.*, 1997). Such a workup should include documentation of external clinical features, X-rays, autopsy, neurohistology investigations, photography and cytogenetic studies (Froster *et al.*, 1997).

Different phenotypic classifications of LMPS were historically suggested. In 1984, Hall proposed three different types based on clinical manifestations, i.e. LMPS without joint fusions, LMPS with fusion of vertebral spinous processes and LMPS with fusion of long bone joint cartilage (Hall, 1984). Several years later, De Smulders *et al.* suggested three LMPS groups, i.e. early onset, late onset and a distinct Finnish type (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990). None of these classifications represent the full pathophysiological, histological, and underlying genetic diversity of LMPS.

Several mechanisms of LMPS pathogenesis were proposed in early case reports, including fragile collagen (Hartwig *et al.*, 2018), primary muscle aplasia (Moerman *et al.*, 1990), and the combination of FADS and jugular lymphatic obstruction (Moerman *et al.*, 1990). In 2002, Cox *et al.* published the most detailed review yet of LMPS related neuromuscular pathology (Cox *et al.*, 2003). This report provided valuable insight into the aetiological diversity of LMPS. Apart from global skeletal muscle atrophy in all cases, the muscle and central nervous system (CNS) neuropathology in their case series were diverse. The findings in Cox's study support the current accepted theory that LMPS is an aetiologically diverse but distinct phenotypic expression of severe and early onset fetal akinesia (Cox *et al.*, 2003).

2.6 Prenatal detection of fetal akinesia and LMPS

Decreased fetal movements are detectable on first trimester prenatal ultrasound but not routinely objectively assessed (Niles *et al.*, 2019). Without a high index of suspicion, close to 75% of fetuses multiple congenital contractures may be missed prenatally (Filges and Hall, 2013). Earlier presentations frequently correlate with increased phenotypic severity, though most cases of fetal akinesia remain undiagnosed until the second or third trimester (Rink, 2011; Niles *et al.*, 2019). Fetal akinesia is most often prenatally diagnosed following the identification of bilateral clubfeet on prenatal ultrasound or fetal evaluation following maternal perception of reduced fetal movements (Filges and Hall, 2013).

Several clinical features of LMPS are visible on first trimester ultrasound, i.e. increased nuchal translucency (NT), subcutaneous oedema or hydrops fetalis, cystic hygroma, fixed flexion of major joints, absent fetal movements, pterygia and a short umbilical cord (Gundogan *et al.*, 2006; Chen, 2012; Niles *et al.*, 2019). During the second and third trimester additional features may include: IUGR, cleft lip or palate, retro- or micrognathia, increased cardiothoracic ratio, absent fetal stomach bubble, scoliosis, pterygia, and a non-vertex presentation (Chen, 2012; Niles *et al.*, 2019). Polyhydramnios due to reduced fetal swallowing is seen in almost all pregnancies progressing beyond the first trimester (Hall, 2014; Niles *et al.*, 2019).

In addition to its diagnostic capability, prenatal ultrasound may provide valuable clues regarding the aetiology of fetal akinesia, prognostication of outcome and aid parental counselling. Improvements in access to high quality prenatal sonography by trained operators is expected to improve detection rates (Niles *et al.*, 2019).

2.7 Genetics of fetal akinesia and LMPS

The application of NGS and bioinformatic analysis is providing novel insights into genes and pathways underlying fetal akinesia and LMPS (Ravenscroft *et al.*, 2011; Hall, 2014; Todd *et al.*, 2015; Beecroft *et al.*, 2018; Pergande *et al.*, 2020). Current wide-spread application of affordable and high-throughput NGS technologies in both

clinical and research settings have been pivotal in improving our understanding of genes and developmental pathways underpinning normal fetal movement (Ravenscroft *et al.*, 2011; Todd *et al.*, 2015; Hall and Kiefer, 2016; Beecroft *et al.*, 2018; Pergande *et al.*, 2020). Like many other phenotypes associated with fetal akinesia, the genetic aetiology of LMPS is heterogenous.

Genes encoding structural components of neuromuscular junction and the fetal acetylcholine receptor (AChR) in particular, are historically linked to both LMPS and non-lethal MPS phenotypes (Missias *et al.*, 1996; Vogt *et al.*, 2008, 2012; Ravenscroft *et al.*, 2011; Beecroft *et al.*, 2018). During embryonic and early fetal life, the AChR consists of 5 subunits (two $\alpha 1$, one $\beta 1$, one γ and one $\delta 1$). An ϵ unit replaces the γ subunit by 33 weeks gestational age and persists into adulthood (Missias *et al.*, 1996). Severe or complete loss of fetal AChR function due to loss of function variants in *CHRNA1* ($\alpha 1$ -subunit), *CHRND* ($\delta 1$ -subunit), *CHRNA1* (γ -subunit), result in an LMPS phenotype (Vogt *et al.*, 2008, 2012). Less severe disruptions with residual receptor function, result in non-lethal phenotypes, e.g. EVMPS, or a myasthenic syndrome later in life (Michalk *et al.*, 2008). Biallelic loss of function variants in *CHRNA1* account for up to 30% of lethal and non-lethal MPS (Morgan *et al.*, 2006; Vogt *et al.*, 2008, 2012).

More recently, genes involved in the post synaptic stabilization, clustering and maintenance of the AChR in the neuromuscular junction, i.e. *RAPSYN*, *MUSK* and *DOK7*, have been linked to several fetal akinesia phenotypes, including lethal FADS and LMPS (Vogt *et al.*, 2008, 2009; Chen, 2012; Tan-Sindhunata *et al.*, 2015). Similarly, variants in genes involved in excitation-contraction coupling (*RYR1*) and sarcomere structure (*NEB*), historically linked to non-lethal myopathies, are now also known to cause LMPS (McKie *et al.*, 2014; Kariminejad *et al.*, 2016; Abdalla *et al.*, 2017).

For all these genes, LMPS results from biallelic loss of function variants, i.e. splice site, nonsense, frameshift, or missense variants predicted to result in either an absence or severe reduction of normal protein function. This supports an autosomal recessive pattern of inheritance in LMPS. Since X-linked genes have been implicated in other fetal akinesia phenotypes and X-linked inheritance described in some LMPS

case reports (Meyer-Cohen J, Dillon A, Pai GS, 1999; Tolmie *et al.*, 2018), an undetermined X-linked genetic cause for LMPS shouldn't be discounted.

These new insights into genotype-phenotype correlations of fetal akinesia, are blurring the boundaries of conditions previously thought to be distinct entities (Beecroft *et al.*, 2018; Hall, Kimber and Dieterich, 2019). Multiple gene domains with different temporal and tissue expression likely contribute to the genetic heterogeneity of fetal akinesia (Hall, Kimber and Dieterich, 2019). Between 2016 and 2019, Kiefer and Hall added an additional 82 novel genes to their gene ontology analysis of arthrogyrosis (Hall and Kiefer, 2016; Kiefer and Hall, 2019). This ontology now includes 402 genes in 29 different groups. Annotation and functional grouping of genes associated with fetal akinesia phenotypes into their respective pathways provide the opportunity for identification and prioritization of novel candidate genes (Kiefer and Hall, 2019).

2.8 Rationale for using WES to diagnose novel causes of fetal akinesia and LMPS

Despite genomic advances and the ever growing number of novel genes linked to fetal akinesia, many patients remain without a genetic diagnosis (Ravenscroft *et al.*, 2011; Pergande *et al.*, 2020). Not only does this prove challenging in providing accurate counselling regarding prognosis and recurrence risks, but remains an obstacle for the development of comprehensive diagnostic genetics tests and therapeutic options (Kiefer and Hall, 2019; Pergande *et al.*, 2020).

Obtaining large quantities of high quality DNA from affected individuals for NGS sequencing often proves challenging in lethal fetal disorders (Beecroft *et al.*, 2018). An alternative approach is parental exome sequencing, in which analysis of rare heterozygous variants in the same gene has had a 52% success rate in diagnosing lethal autosomal recessive fetal disorders (Ellard *et al.*, 2015). This approach could be adapted for X-linked disorders but would miss de novo disease causing variants in the fetus.

Using a targeted gene panel approach to identify pathogenic variants in known fetal akinesia or LMPS disease genes is useful and perhaps more affordable than WES or WGS but will miss variants in novel disease genes. In contrast to WES, WGS offers genomic coverage of both protein-coding and non-coding regions with the additional benefit of longer sequence reads facilitating determination of copy number variation (Beecroft *et al.*, 2018). However, WGS often remains prohibitive in resource constrained settings due to cost, volume, and complexity of generated data (Beecroft *et al.*, 2018).

The application of WES and bioinformatic analysis to diagnose prenatally lethal conditions, a so called “molecular autopsy”, has proven diagnostic utility (Shamseldin, Swaid and Alkuraya, 2013; Ellard *et al.*, 2015; Quinlan-Jones *et al.*, 2019). An approach which includes a combination of ‘deep clinical phenotyping’ with trio WES and CNV analysis, has the ability to increase detection rates of disease-causing variants in fetal akinesia from 41 to 73% (Pergande *et al.*, 2020).

2.9 Phenotyping in the genomics era

Precise and comprehensive documentation of observable individual characteristics and traits, or phenotyping, has long been an integral component to both clinical practice and research in medical genetics (Robinson, 2012). The advent of large international phenotypic and genomic databases has necessitated the use of a standardized vocabulary by clinicians and researchers to facilitate the interpretation and comparison of vast amounts of complex data (Robinson *et al.*, 2008; Köhler *et al.*, 2019). The Human Phenotype Ontology (HPO) was established in 2008 and provides such a standardized nomenclature (Robinson *et al.*, 2008).

In recent years, the concept of ‘deep phenotyping’ has evolved. ‘Deep phenotyping’ encompasses not only observable clinical features, but incorporates and integrates all phenotypic traits of an individual, including physiologic (e.g. biochemical and microscopic) and imaging (photographic and radiologic) data (Robinson, 2012). The fusion of ‘deep phenotyping’ with genomics is paving the way to establishing new

genotype-phenotype correlations, precision medicine techniques and gene directed therapies (Yehia and Eng, 2019).

2.10 Conclusion

LMPS is a rare and phenotypically distinct presentation of severe and early onset fetal akinesia. Data regarding the prevalence, phenotype, and genetic aetiology of LMPS in Southern Africa is non-existent. Recent and ongoing genomic advances are greatly improving our understanding of genes and developmental pathways underlying fetal akinesia and LMPS. The underlying genetic causes of LMPS are heterogenous and remain undetermined for some cases. Founder effects for lethal fetal akinesia phenotypes are known to occur in several international populations. An NGS approach combined with 'deep phenotyping' provides the highest probability of solving the genetic aetiology of prenatally lethal genetic disorders, like LMPS.

3. Research Methodology

3.1 Study Setting

Tygerberg Hospital is a tertiary level referral hospital, serving a population of approximately 2.6 million people in the Western Cape. Tertiary obstetric and medical genetic services of the province are split between Tygerberg Hospital and Groote Schuur Hospital. Tygerberg Hospital's drainage area includes the Cape Town Metro East (Northern, Eastern, Tygerberg and Khayelitsha subdistricts), and rural (Cape Winelands, West Coast and Overberg) districts.

The Clinical Unit of Medical Genetics and Genetic Counselling based at Tygerberg Hospital provides a multidisciplinary specialist genetic service which includes a weekly fetal anomaly and genetic disorders counselling clinic in conjunction with the Fetal Medicine unit. The prenatal ultrasound, fetal medicine and obstetric service functions as a level II and III referral unit for midwife obstetric units and district-based sonography services, as well as for level I and level II hospitals. In the metropolitan drainage area, there are between 30 000 - 35 000 deliveries (live and stillbirth >500g) reported annually, and in the rural districts approximately 18 000 deliveries (Perinatal Problem Identification Program data, 2016; personal communication Prof S. Gebhardt).

Women attending the Tygerberg Hospital Fetal Medicine unit are generally at increased risk of obstetric or medical complications requiring specialist fetal ultrasonography, or have an increased risk of fetal aneuploidy, a suspected fetal anomaly, teratogen exposure during pregnancy or a personal or family history indicating a possible genetic disorder.

3.2 Study Population

All fetuses of women attending the prenatal ultrasound and/or obstetric services at Tygerberg Hospital and who meet the case definition of LMPS (See **Section 3.3.1**) are included in this study. Persons of all ethnicities are represented in the patient

population attending Tygerberg hospital, though the majority are of 'Coloured South African' and 'Black South African' ancestry (South African Census terms, 2011).

3.3 Study Design

To meet the study aims and objectives, the research project was divided in two parts, i.e. a descriptive case series of retrospective and prospective LMPS cases presenting to Tygerberg Hospital over an 8-year period and an initial genomic investigation and analysis of LMPS using WES and various bioinformatic and variant interpretation tools.

3.3.1 Descriptive case series

A case definition for LMPS was established from the literature review. Features of LMPS that are present in all fetuses, independent of gestational age, were identified. To meet the case definition of LMPS for inclusion in our case series, a fetus must have had multiple congenital joint contractures with pterygia of all major joints in both the upper and lower limbs and be non-viable either prenatally or in the peripartum period.

Retrospective cases (2011-2015) meeting the case definition of LMPS were identified from review of medical records, databases, and Perinatal Problem Identification Programme (PPIP) data kept by the medical genetics, obstetric and prenatal ultrasound units. Prospective cases (2016-2018) meeting the case definition were identified either prenatally on obstetric ultrasound or following delivery on clinical examination by a medical geneticist or medical genetics registrar at Tygerberg Hospital.

3.3.1.1 Inclusion criteria:

Retrospective and prospective cases presenting to Tygerberg Hospital that meet the case definition of LMPS, i.e. prenatal onset of severe fetal akinesia as evidenced by the presence of all the following features:

- multiple congenital joint contractures of upper and lower limbs with
- pterygia of all major joints in the upper and lower limbs

- non-viability or inevitable lethality either prenatally or peripartum

3.3.1.2 Exclusion criteria

- Insufficient data or evidence to meet the case definition of LMPS

3.3.2 Genomic investigation and analysis using WES and bioinformatics

Prospective cases meeting the LMPS case definition are eligible for further genomic investigation by enrolment in a separate research study within the Clinical Unit of Medical Genetics and Genetic counselling entitled, “*Translational research - the use of genomic testing, especially whole exome sequencing, for diagnostic purposes in SA*” (HREC reference: N18/03/031). This study protocol provides for enrolment of individuals with a variety of rare mendelian disorders (MD) in whom standard or conventional genetic testing did not identify a genetic cause. Established protocol procedures for informed consent, data collection and analysis were followed. The same inclusion and exclusion criteria applied, and existing informed consent forms were used.

3.3.2.1 Inclusion criteria

- Participants of any age (including postmortem samples) with a strongly suspected MD on grounds of history, family history, clinical examination and/or investigations where standard/conventional genetic testing available in the state sector in South Africa has been performed and has not resulted in a diagnosis.
- Where further genomic testing, especially WES is an appropriate further investigation e.g. MD with non-specific phenotype, or condition known to be very genetically heterogeneous, or alternative test methods not readily available.

3.3.2.2 Exclusion criteria

- Relevant standard of care/conventional genetic investigations available in the state sector in South Africa has not been performed yet
- Patient has a confirmed molecular genetic diagnosis on conventional genetic testing
- Phenotype not consistent with a MD
- WES or NGS gene panel not considered an appropriate test for technical or clinical reasons
- Family unable to commit to the follow-up genetic counselling or the results delivery process

3.3.2.3 Selection of a suitable trio for genomic investigation

The proband (affected fetus) and both parental DNA samples were included for trio analysis using WES. The use of family-based trios or quads significantly improves the success rate of identifying disease-causing variants compared to analysing the exome of the proband alone.

Given the cost of NGS technologies, including WES, and limited research budget available for this project, an initial trio (fetus affected with LMPS and both unaffected parents) was selected from prospective LMPS cases and consented for further genomic investigation and analysis.

To ensure the highest probability of successful WES and identification of disease-causing variants, the following criteria were used to select the most suitable initial trio:

- Availability and willingness of both parents to consent to inclusion in the case series and further genomic analysis
- Availability of fetal and both parental DNA samples
- Quantity and quality of extracted DNA from blood samples sufficient for WES
- Completeness of phenotypic data supporting the diagnosis of fetal LMPS, i.e. prenatal ultrasound, clinical examination, autopsy and fetal radiography
- Families with more than one fetus or pregnancy affected by LMPS (if available)

3.4 Data Collection

3.4.1 Clinical data collection

3.4.1.1 Retrospective cases (2011-2015)

The following sources were used to identify cases retrospectively and compile family history, clinical and phenotypic data of those matching the case definition from LMPS:

- Perinatal mortality records and PPIP data kept by the medical genetics and obstetric departments:
 - Fetuses delivered at Tygerberg Hospital due to miscarriage, medical termination of pregnancy (TOP), stillbirth, or early neonatal death (ENND) in labour ward, are examined daily by a medical geneticist or medical genetics registrar. A perinatal mortality document is completed for all cases and used for capturing PPIP data. The document includes birth weight, sex, dysmorphic features, and other findings on external examination of the fetus, documented in free text. Possible diagnoses, further investigations, and recommendations are noted. Copies of these documents are kept by medical genetics for record purposes.
- Prenatal ultrasound database (Astraia ®):
 - Astraia ® is a database of all formal prenatal ultrasounds performed at the prenatal ultrasound and fetal medicine unit. Information captured includes maternal demographic, health, and pregnancy data, fetal growth, soft markers and anomalies detected on prenatal ultrasound.
- Medical genetics prenatal counselling database
 - All prenatal cases counselled by members of the medical genetics team are captured in an electronic database on a password protected departmental computer.

- Electronic hospital records
 - Tygerberg Hospital keeps an online, password protected electronic record of all hospital patients' medical records. Once LMPS cases are identified, more clinical information is extracted from the electronic hospital records
- Laboratory results
 - DISA lab (pre-2015) and Trakcare (2015-present) are the official National Health Laboratory Service result viewing tools. These were used to retrieve results of investigations done as part of standard of care either prenatally or postnatally e.g. chromosome analysis, placental histology, and autopsy results.
- Radiology
 - X-rays (fetograms) of affected fetuses, if obtained, were reviewed for radiological anomalies using the hospital's radiology viewer, iSite Enterprise.

3.4.1.2 Prospective cases (2016-2018)

Prospective cases meeting the definition of LMPS were identified either:

- Prenatally when features of LMPS are evident on prenatal ultrasound and/or
- Following delivery on clinical examination by a medical geneticist or medical genetics registrar.

Following identification of a prospective case, informed consent for inclusion in the case series accompanied by a detailed family history and genetic counselling were provided directly to the woman and her partner, wherever possible. We attempted to obtain X-rays, consent for photographs, autopsy, and tissue samples for DNA for all prospective cases.

3.4.2 Genomic investigation using WES

Following the pre-test genetic counselling session and informed consent from both parents and/or informative siblings of an affected fetus, the following data collection procedures were followed:

3.4.2.1 Clinical data collection

Family history, clinical and phenotypic data were available from the data collected for the case series. Any relevant gaps in information relating to family and pregnancy history were addressed during the pre-test counselling session.

3.4.2.2 Blood collection

Up to 5 ml of blood was drawn from each parent by means of peripheral venous puncture. Fetal blood was obtained from either cord blood sampling or postmortem cardiocentesis by an experienced clinician.

3.4.2.3 DNA extraction

Fetal DNA was extracted and purified from a tissue sample of consented participants using standard protocols within the Division of Molecular Biology and Human Genetics at Stellenbosch University (SU). Samples were prepared by postgraduate students within the Division working under supervision of Dr Caitlin Uren.

3.4.2.4 Whole exome sequencing

The Central Analytical Facility at SU conducted WES using Ion Torrent™ semiconductor sequencing technology. The Ion AmpliSeq™ Exome RDY Kit provided exome enrichment and high coverage of >90% of targeted bases at 20x sequencing depth. The proband and parental samples were included in the same run, ensuring cost-effective trio sequencing. Large amounts of raw exome sequencing data were generated, stored, and backed up at SU.

3.5 Data Analysis

3.5.1 Clinical data analysis

Both pedigree and phenotypic data informed further interpretation of genomic data. Family history and pedigrees were assessed to identify patterns of inheritance. Deep phenotyping was facilitated by the extraction of individual phenotypic features from prenatal ultrasound, external examination of the fetus, radiography, microscopic and macroscopic postmortem findings. Where parents provided consent for photography, photographs were used in conjunction with phenotypic data from clinical records for comprehensive phenotyping.

Phenotypic features were reported according to the Human Phenotype Ontology (HPO). The HPO provides a standardised vocabulary for phenotyping that has become an invaluable resource in the phenotype-driven analysis of NGS data (Robinson *et al.*, 2008; Groza *et al.*, 2015). Since its introduction in 2008, the HPO has become the standard nomenclature for deep clinical phenotyping in rare disease research (Köhler *et al.*, 2019). The HPO is freely available at www.human-phenotype-ontology.org.

3.5.2 Genomic data analysis and the bioinformatics pipeline

3.5.2.1 The bioinformatics pipeline

Exome sequencing data were analysed under supervision of Dr Caitlin Uren using an existing bioinformatic pipeline for multiple NGS projects within the Division of Molecular Biology and Human Genetics, SU. The first step in the pipeline mapped our exome sequence data to reference sequences using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). The Genome Analysis Toolkit (GATK) (McKenna *et al.*, 2010) realigned these sequences around indels and recalibrated quality scores. Reads not mapped to the reference sequence were removed with SamTools (Li *et al.*, 2009). SamTools and GATK were used for variant calling and generated Variant Call Format (VCF) files.

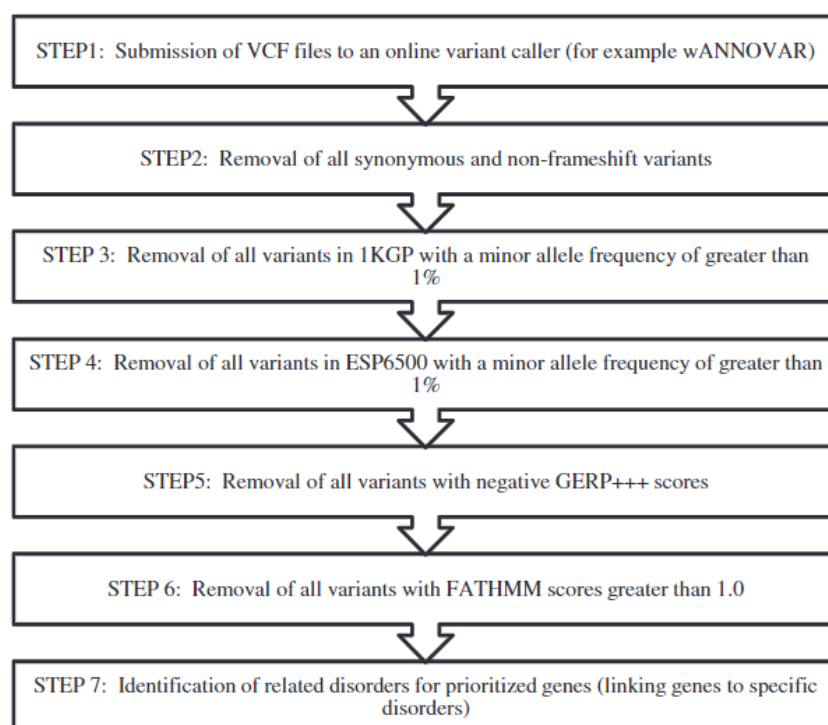
3.5.2.2 Variant annotation and filtering using standardised tools

Due to the vast amount of variation in individual exomes, variant annotation and filtering was an essential step to order and narrow the number of variants prior to further interpretation and classification. Variant annotation and filtering allowed the investigator to:

- remove polymorphisms present in population databases e.g. gnomAD (Karczewski *et al.*, 2020)
- remove synonymous and non-frameshift variants which do not alter the amino acid and ultimate protein product
- assess variants by expected effect on transcription and translation, e.g. frame shift, nonsense, splice site or missense
- identify SNVs that are usually highly conserved across different species, e.g. GERP score calculation (Davydov *et al.*, 2010)
- examine variants in genes known to be associated with the disease or phenotype, e.g. OMIM and Pubmed search
- examine variants in genes interacting with the known genes in a common pathway, e.g. STRINGdb (Szklarczyk *et al.*, 2019)
- submit variants of interest to computational and pathogenicity prediction tools, e.g. UniProt (Venselaar *et al.*, 2010), FATHMM (Shihab *et al.*, 2014), SIFT (Sim *et al.*, 2012), CADD (Rentzsch *et al.*, 2019)
- assess, filter, and compare variants according to zygosity, which is useful when the inheritance pattern of the condition is known and where sequencing is performed in informative family members

Variants were filtered using Taper™ (Glanzmann *et al.*, 2016) and Varseq®. TAPER™ is a free tool developed at SU which implements a set of seven logical steps by which to filter and prioritize candidate variants that could be associated with disease (**Figure 2**). The tool is aimed for implementation in laboratories with limited bioinformatics capacity and can be setup on a Windows operating system without any programming knowledge required. Varseq® is a commercially available variant annotation and filtering software product from leading bioinformatics and biomedical research company, Golden Helix Inc. A trial version of this efficient and user-friendly tool was accessed to compare variants with those filtered through TAPER™.

Figure 2: The seven-level filtration framework and backbone of TAPER™ (Glanzmann *et al.*, 2016)



3.5.2.3 Variant interpretation and classification

During variant annotation and filtering narrowing of the variants of interest occurred, however given the wide genetic heterogeneity of fetal akinesia, a significant number of variants remained. The following approach to prioritize the assessment of variants of interest was followed:

- Step 1: Assess variants in genes known to cause LMPS
- Step 2: Assess variants in genes known to cause broader fetal akinesia phenotypes
- Step 3: Assess variants in genes in the same pathway as genes known to cause LMPS and broader fetal akinesia phenotypes
- Step 4: Assess variants of interest in genes not currently known to cause LMPS, fetal akinesia or interact with other genes in their known pathways.

Variants of interest were assessed with regards to pathogenicity and classified according to the principles outlined in the American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG-AMP) guidelines of 2015 (Richards *et al.*, 2015). These guidelines establish a common framework for variant classification and recommends the use of specific standard terminology to describe variants identified in known morbid genes that cause MD, i.e. "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," and "benign". The evidence framework (Figure 3), describes the process of classifying variants into these five categories based on standardised criteria using typical types of variant evidence e.g. population, computational, predictive, functional and segregation data. These guidelines are not intended for classification of variants in candidate genes with no known disease association, so called "genes of uncertain significance".

Figure 3: Evidence framework for assigning pathogenicity from the 2015 ACMG-AMP guidelines (Richards *et al.*, 2015)

	Benign		Pathogenic			
	Strong	Supporting	Supporting	Moderate	Strong	Very strong
Population data	MAF is too high for disorder BA1/BS1 OR observation in controls inconsistent with disease penetrance BS2			Absent in population databases PM2	Prevalence in affecteds statistically increased over controls PS4	
Computational and predictive data		Multiple lines of computational evidence suggest no impact on gene /gene product BP4 Missense in gene where only truncating cause disease BP1 Silent variant with non predicted splice impact BP7 In-frame indels in repeat w/out known function BP3	Multiple lines of computational evidence support a deleterious effect on the gene /gene product PP3	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before PM5 Protein length changing variant PM4	Same amino acid change as an established pathogenic variant PS1	Predicted null variant in a gene where LOF is a known mechanism of disease PVS1
Functional data	Well-established functional studies show no deleterious effect BS3		Missense in gene with low rate of benign missense variants and path. missenses common PP2	Mutational hot spot or well-studied functional domain without benign variation PM1	Well-established functional studies show a deleterious effect PS3	
Segregation data	Nonsegregation with disease BS4		Cosegregation with disease in multiple affected family members PP1	Increased segregation data →		
De novo data				De novo (without paternity & maternity confirmed) PM6	De novo (paternity and maternity confirmed) PS2	
Allelic data		Observed in <i>trans</i> with a dominant variant BP2 Observed in <i>cis</i> with a pathogenic variant BP2		For recessive disorders, detected in <i>trans</i> with a pathogenic variant PM3		
Other database		Reputable source w/out shared data = benign BP6	Reputable source = pathogenic PP5			
Other data		Found in case with an alternate cause BP5	Patient's phenotype or FH highly specific for gene PP4			

3.5.2.4 Incidental findings

During the search for genetic variants which may cause LMPS (primary variants), unexpected or unrelated variants (incidental or secondary findings) may be detected. Incidental findings are unanticipated, whereas secondary findings are actively sought but not associated with the indication for the test (Weiner, 2014). In our research, we did not actively seek or report on secondary findings. Our aim was to identify primary variants associated with LMPS.

The ACMG released updated recommendations for reporting incidental findings during exome and genome sequencing in clinical diagnostic settings (Kalia *et al.*, 2017). These recommendations are therefore not necessarily applicable to genomic research. The ACMG recommends the reporting of pathogenic variants identified in 59 selected genes since the associated conditions are actionable, i.e. morbidity and/or mortality of the associated disease may be alleviated through early screening or treatment. The ACMG recommends that patients be given the choice to 'opt out' of receiving information on incidental findings and that they should understand the consequences of doing so. The option of receiving incidental findings was discussed with participants consenting to genomic investigation and their choice indicated on the consent form. Confirmatory testing and feedback are offered for incidental variants in participants opting to receive this information.

3.6 Ethics approval and other ethical considerations

The case series was approved by the Health Research Ethics Committee (HREC) of SU (HREC reference: C19/06/017). A waiver of informed consent for the inclusion of retrospective cases was approved. Informed consent for inclusion in the case series was obtained, wherever possible, from prospective cases using the "Consent Form for Case Reports" of Stellenbosch University, Version 1, 2008.

HREC approval was obtained for the existing genomics investigation study protocol, "Translational research- the use of genomic testing, especially whole exome sequencing, for diagnostic purposes in SA" (HREC reference: N18/03/031).

Participants enrolled in the genomics study were consented using the study's approved informed consent forms.

3.6.1 Data management and privacy

The study adhered to the ethical guidelines set out in the Declaration of Helsinki, 2013. As in any research study, the privacy of participants is of great importance, however genomic data can never be truly anonymised in populations using publicly accessible genealogical databases (Gymrek *et al.*, 2013). While this is likely not the case in the population accessing our service, it is important to minimise the possibility that research participants are identified.

All participant data (clinical and genomic) are kept strictly confidential and were de-identified by a numerical study identifier. A participant identification log was kept as a separate document in a secure location by the investigator. De-identified participant information was entered into an electronic password protected study database on a password protected computer. The password is only known to the investigator.

There was no deviation from standard of care practices when collecting data for the case series. Where prospective cases were identified, consent for inclusion in the case series and use of photographs was obtained. A waiver of consent was obtained from the SU HREC for retrospective cases included in the case series.

3.6.2 Genetic counselling and informed consent for genomic investigation

WES is a powerful genomic test which generates large volumes of information. Written consent was required for each participant. In the case of a fetus, the mother provided consent. Refusal to take part in the research study did not impact on their clinical management and care.

Careful attention was paid to both pre-test and post-test counselling of participants in whom further genomic testing was performed. The pre-test counselling session included a detailed family history and drawing of a three-generation pedigree. The

benefits and limitations of WES, as well as the family's expectations and concerns regarding their involvement in the study were addressed. The possibility of detecting incidental findings and whether they should be disclosed were discussed. Participants indicated whether their clinical and genomic data should be stored or destroyed following completion of the genomic investigation.

3.6.3 Storage of genomic data

Genomic data is stored on a password protected database for participants consenting to data storage. Data of patients who did not consent to continued storage, will be deleted.

3.6.4 Risk to study participants: blood sample collection

There is a minimal risk associated with having a peripheral venous blood sample taken, namely a small risk of bruising or local inflammation. To minimize risk and discomfort, blood samples were collected by experienced clinical staff. Collecting fetal blood from cord blood or postmortem cardiocentesis were discussed with the parents. These procedures did not carry any additional risk of harm to the fetus who had demised.

3.6.5 Benefits to study participants

Participants in the case series may not benefit directly from the study. Parents of previously undiagnosed LMPS cases identified during the study, would benefit from receiving a diagnosis and genetic counselling regarding recurrence risk. This information could provide valuable information for the management of future pregnancies. Should genomic investigations reveal the presence of validated pathogenic or disease-causing variants, these could potentially be used for prenatal diagnosis in subsequent pregnancies.

4. Results: Descriptive Case series

4.1 Results overview

The case series included 25 fetuses that met the case definition of LMPS. These 25 fetuses are from 24 pregnancies in 20 women. Between 2011 and 2015, 12 cases were identified retrospectively, whereas 13 cases were identified prospectively between 2016 and 2018 (**Table 1**). Sex distribution amongst affected fetuses was similar, namely 10 males and 11 females. In 4 cases the fetal sex remained undetermined due to early fetal gestation or maceration.

Table 1: Number of LMPS cases per year with sex distribution

	Year	Total (n)	Fetal sex		
			Male	Female	Unknown
Retrospective cases (n = 12)	2011	1	0	1	0
	2012	1	0	1	0
	2013	2	2	0	0
	2014	4	2	2	0
	2015	4	2	2	0
Prospective Cases (n = 13)	2016	3	1	1	1
	2017	6	2	3	1
	2018	4	1	1	2
Total		25	10	11	4

An overview of parental demographic, maternal health, pregnancy outcomes, and LMPS recurrence is represented in **Table 2**.

4.2 Demographic information

Mean maternal age was 26.5 (range 16-38) years. 90% of women were of black African ancestry (5 Xhosa and others unspecified). The ancestry of 2 women was

Table 2: Overview of women (M01-M20) and affected fetuses (F01-F25)

Women	M01	M02	M03		M04		M05	M06	M07	M08	M09	M10	M11	M12	M13	M14			M15		M16	M17	M18	M19	M20
Affected Fetus	F01	F02	F03	F13	F04	F11	F05	F06	F07	F08	F09	F10	F12	F14	F15	F16	F17	F22	F18	F25	F19	F20	F21	F23	F24
Year(s)	2011	2012	2013	2016	2013	2015	2014	2014	2014	2014	2015	2015	2015	2016	2016	2017	2017	2018	2017	2018	2017	2017	2017	2018	2018
Fetal sex	F	F	M	U	M	M	F	M	F	M	F	F	M	M	F	M	U	U	F	U	F	M	F	F	M
Maternal age (years)	27	19	23	26	20	21	23	35	38	33	31	29	21	26	34	31	31	32	28	29	16	21	23	24	22
Gravidity	2	1	1	2	1	2	1	4	3	3	2	2	1	2	3	3	3	4	2	3	1	1	1	6	1
Parity	1	0	0	0	0	0	0	3	2	1	1	1	0	1	1	2	2	2	1	1	0	0	0	3	0
Pregnancies with LMPS (n)	1	1	2		2		1	1	1	1	1	1	1	1	1	2			2		1	1	1	1	1
Fetuses with LMPS (n)	1	1	2		2		1	1	1	1	1	1	1	1	1	3			2		1	1	1	1	1
Miscarriage (other than known LMPS)	0	0	0	0	0	0	0	0	0	1 (T2)	0	0	0	0	1 (T1)	0	0	0	0	0	0	0	0	2 (T1)	0
Ancestry (mat)	B	U	B		B		B	B	B	U	B	B	B	B	B	B			B		B	B	B	B	B
Ancestry (pat)	U	U	U		U		U	U	U	U	U	U	U	U	U	B			U		U	U	B	B	B
Maternal comorbidities	-	-	-	-	CHPT		HIV	-	CHPT	-	-	HIV	-	HIV	-	HIV			-	-	-	-	-	-	MDE
Pregnancy complications	-	-	-	-	-	-	-	-	-	-	ASHPT amnio drain	-	-	-	-	-	-	-	-	-	CS	RPOC	PE CS	CS	-
Singleton/Twin	S	S	S	S	S	S	S	S	S	S	DCDA	S	S	S	S	DCDA		S	S	S	S	S	S	S	S
Pregnancy outcome	IUD	MTOP	IUD	TOP	STOP	MTOP	MTOP	MTOP	IUD	MTOP +FC	IUD	MTOP	MTOP	IUD	IUD	MTOP	IUD	MTOP	IUD	STOP	ENND	MTOP	IUD	ENND	MTOP
Method of delivery	V	V	V	U	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	CS	V	CS	CS	V
Gestation at delivery (completed weeks)	27	19	17	13	14	20	23	23	30	27	34	19	25	26	28	21	21 demised at 13	12	25	15	28	23	34	28	22

Table 2 Abbreviations: ASHPT – acute severe hypertension, B Black African, CHPT- chronic hypertension, CS – caesarean section, DCDA – dichorionic diamniotic pregnancy, FC – feticide, HIV – human immunodeficiency virus, IUD – intrauterine death, MDE – major depressive episode, MTOP – medical termination of pregnancy, PE -preeclampsia, RPOC – retained products of conception, S - Singleton, STOP – surgical termination of pregnancy, T1 – first trimester, T2 second trimester, U Unknown, V – vaginal

unknown. Paternal ancestry was often not documented, though where reported (4/4) fathers were of black African ancestry (3 Xhosa and 1 Swazi). No women reported a history of consanguinity with the father of the fetus. Apart from LMPS in their own offspring, there was no broader family history of neuromuscular disease in any women.

4.3 Maternal health

45% of women were primigravid at their index presentation of a pregnancy affected by LMPS. Maternal comorbidities included HIV infection (4/20), chronic hypertension (2/20) and major depressive disorder during pregnancy (1/20). One woman developed preeclampsia (PE) at 34 weeks, and another woman presented at the same gestation with acute severe hypertension (ASHPT) without proteinuria in a twin pregnancy discordant for LMPS.

4.4 Pregnancy outcomes and complications

Pregnancy outcomes are classified into 3 groups, namely termination of pregnancy (TOP), spontaneous intrauterine death or stillbirth and early neonatal death. These outcomes are represented in **Table 3**.

4.4.1 Termination of pregnancy

TOP was performed in 56% (14/25) of cases. A medical TOP was performed in 10 cases with an average gestational age of 20 completed weeks. A medical TOP with feticide procedure was performed in 1 case (F08) due to an advanced gestation of 27 weeks. Surgical TOP was performed in 2 cases (F04 and F25) with an average gestational age of 14 completed weeks. In one case (F13) the method of TOP is unknown since the woman was referred to a regional hospital for further management of the TOP procedure.

Table 3: Pregnancy outcomes and gestational age

Pregnancy outcome	Fetuses (n)	Gestational age Mean (weeks)	Gestational age Range (weeks)
TOP: Total number	14	19	12-27
TOP: medical no feticide	10	20	12-25
TOP: medical and feticide	1	27	27
TOP: surgical	2	14	13-15
TOP: method unknown	1	13	13
IUD/Stillbirth (Apgar 0)	9	26	13-34
ENND (Apgar recordable)	2	28	28
Total	25	22	12-34

4.4.2 Spontaneous intrauterine death or stillbirth

Spontaneous intrauterine death (IUD) was diagnosed in 36% (9/25) of fetuses at a mean gestational age of 26 completed weeks. In 5 cases, IUD was detected at first presentation to Tygerberg Hospital (F03, F07, F15, F17 and F18). Following the recognition of poor fetal prognosis at 23 weeks, one woman opted to continue the pregnancy (F14) but subsequently presented with a spontaneous IUD at 26 weeks. Another opted to return for TOP and feticide procedure after two days and presented with a spontaneous IUD on the day of the scheduled TOP (F01). In 2 cases (F09 and F21) fetal heart activity was present during labour, but both fetuses were stillborn with no signs of life and Apgar scores recorded as 0, 0, 0.

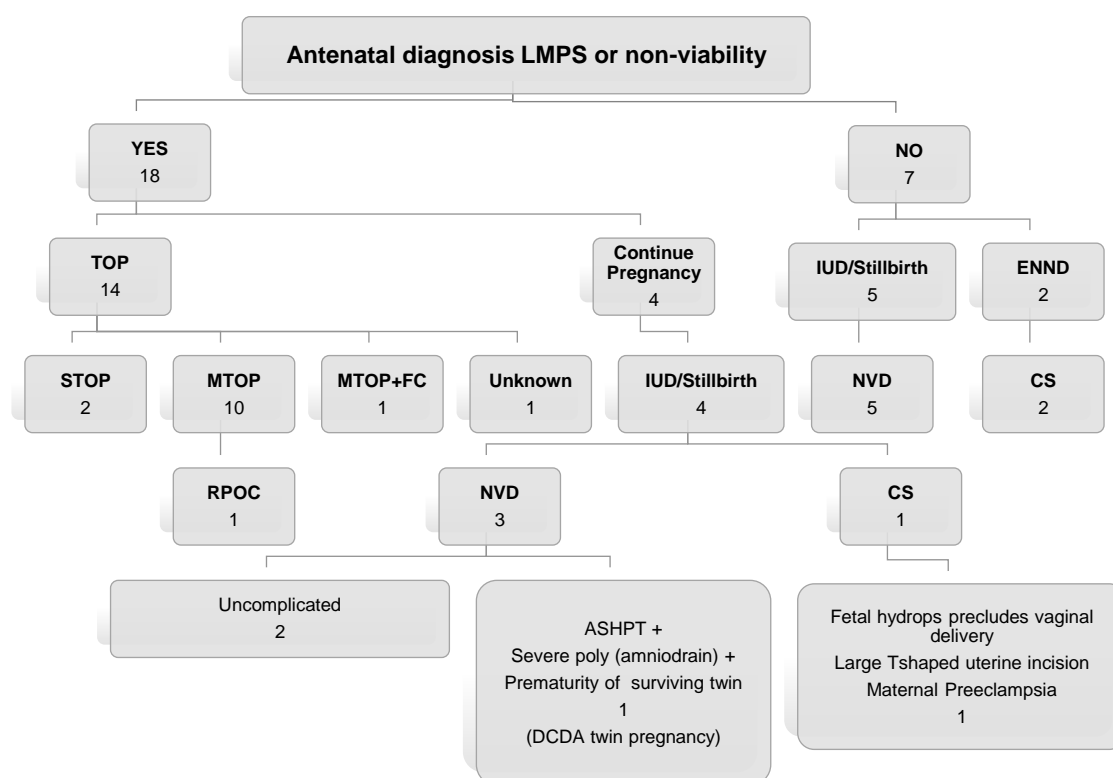
4.4.3 Early neonatal death

Two fetuses (F19 and F23) showed signs of life following delivery via Caesarean section but demised shortly after delivery despite resuscitative efforts. Apgar scores were recorded as 1, 2, 1 and 1, 1, 1, respectively. In both cases the diagnosis of LMPS was unknown antenatally and the poor outcome unexpected. Both women initiated antenatal care late, did not attend regular antenatal care and had no formal ultrasound assessment. They each presented with preterm labour at an estimated gestational age of 28 weeks. Caesarean section was performed in both cases for non-reassuring fetal condition as detected on cardiotocography during labour.

4.4.4 The relationship between antenatal detection of LMPS, pregnancy outcome and complications

LMPS or non-viability due to hydrops fetalis or pulmonary hypoplasia were diagnosed antenatally in 72% (18/25) of fetuses. In 28% (7/25), the diagnosis was either unknown or fetal non-viability not detected antenatally. **Figure 4** summarises the relationship between prenatal detection of LMPS or fetal non-viability with pregnancy outcomes, mode of delivery and associated complications. Where LMPS or non-viability of the fetus was recognized antenatally, 78% (14/18) of women opted for TOP. The only known complication related to TOP was retained products of conception following MTOP in M17. The remaining 4/18 women opted to continue their pregnancy which ended with either an IUD or stillbirth.

Figure 4: The relationship between prenatal detection of LMPS or fetal non-viability with pregnancy outcomes, mode of delivery and associated complications.



3/4 women who opted to continue their pregnancy delivered vaginally and 1/4 had a Caesarean section. 2/3 vaginal deliveries were uncomplicated with no documented maternal peripartum complications. One woman (M09), with a DCDA pregnancy

discordant for LMPS, developed ASHPT and severe polyhydramnios requiring amniotic fluid drainage at 33 weeks and delivered vaginally at 34 weeks. Another woman (M18) who opted to continue her pregnancy developed preeclampsia with severe hydrops fetalis at 34 weeks gestation. The severe fetal hydrops in the live fetus precluded vaginal delivery and a Caesarean section was performed knowing that the fetus is non-viable and that no resuscitative attempts would be attempted. The Caesarean section incision had to be extended to a larger T-incision to facilitate delivery of the fetus. The fetus had no signs of life at delivery with Apgar scores of 0, 0, 0.

Where LMPS or non-viability was not detected antenatally, 71% (5/7) women had an IUD or stillbirth with uncomplicated vaginal deliveries. Caesarean sections for non-reassuring fetal condition were performed in 2 cases (F19 and F23). In both cases the affected neonates demised shortly following delivery despite resuscitative efforts.

4.4.5 Pregnancy outcome in twin pregnancies

Most pregnancies were singleton (92%). There were two dichorionic diamniotic (DCDA) twin pregnancies. In one of these, both twins (F16 and F17) were affected by LMPS and in the other the twins were discordant for LMPS (F09).

In the pregnancy with both affected twins, F17 died in utero at 13 weeks gestational age, as estimated by crown rump length. F16 was alive at first formal ultrasound at 21 weeks gestational age. The woman opted for medical TOP of the pregnancy. Following delivery, clinical and radiographic features of LMPS were evident in both fetuses. F16 was male whilst the sex of F17 remained undetermined due to early gestation and fetal maceration.

In the twin pregnancy discordant for LMPS (F09), the woman elected to continue with the pregnancy. The pregnancy was subsequently complicated by severe polyhydramnios, necessitating amniotic fluid drainage at 33 weeks. Spontaneous preterm labour occurred at 34 weeks with concurrent ASHPT necessitating delivery. Both twins were female and delivered vaginally by forceps. There were no signs of life

in the twin affected by LMPS at delivery with Apgar scores recorded as 0, 0, 0. The normal twin had a stormy neonatal course and severe neonatal jaundice requiring exchange transfusion.

4.5 Recurrence of LMPS

20% (4/20) of women had a proven recurrence of LMPS in a following pregnancy. Another 3/20 women had at least one other pregnancy loss or miscarriage, though information was either unavailable or insufficient to attribute the loss to LMPS. Recurrence always occurred with the same partner. No woman had a proven recurrence of LMPS with a different partner. One woman who had a recurrence with the same partner, had a subsequent healthy pregnancy with a different partner. All women with a proven recurrence, presented for tertiary prenatal ultrasonography of which two occurred in the first trimester (gestational age range 12⁺² – 18⁺³ weeks). All accepted TOP following confirmation of LMPS recurrence on prenatal ultrasound.

4.6 Prenatal ultrasound findings

Prenatal ultrasound examinations were performed at Tygerberg Hospital's Fetal Medicine unit for 76% (19/25) of fetuses. First trimester assessments were performed for 3/19 (16%), second trimester fetal anatomy assessment prior to 24 weeks for 11/19 (58%) and late second or third trimester ultrasound examinations in 5/25 (26%). Ultrasounds outside the prenatal ultrasound unit but within Tygerberg Hospital, i.e. in obstetric admissions, labour ward, or obstetric outpatients were performed in 5 cases.

4.6.1 First trimester NT ultrasound findings

First trimester NT ultrasound examinations were performed in 3 pregnancies. Two of these were for women with LMPS recurrence. All fetuses were alive at the time of the ultrasound. Ultrasound findings on NT scan are reflected in **Table 4**. In all three ultrasounds an increased NT (range 3.9 mm - 12.88 mm), generalised oedema and/or hydrops fetalis, reduced or absent fetal movements and abnormal flexion or positioning of major joints were detected. Additional findings included cystic hygroma

and megacystis in one fetus and a single umbilical artery (SUA), with small stomach bubble and small bladder in another. Pterygia were not reported on any first trimester ultrasounds.

Table 4: First trimester NT ultrasound findings

Affected Fetuses	F04	F13	F22
LMPs Recurrence	-	+	+
Gestation (weeks)	12.6	13.4	12.2
Live fetus	+	+	+
Fetal movements	reduced	absent	reduced
Increased NT	+	+	+
NT measurement (mm)	3.9	12.88	7.4
Oedema	+	+	+
Hydrops fetalis	-	+	+
Cystic hygroma	+	-	-
Abnormal joint flexion	+	+	+
Pterygia	-	-	-
Hands	-	abnormal position	-
Feet	-	abnormal position	-
Micrognathia	-	-	-
Chest and Cardiac	-	-	-
GIT	-	small stomach bubble	-
Urinary tract	megacystis	-	-
Scoliosis	-	-	-
Soft markers and other anomalies	reversed a-wave in ductus venosus	-	-
Amniotic fluid	N	oligohydramnios	N
Cord	N	single umbilical artery	N
Placenta	N	N	N

4.6.2 Second trimester (fetal anatomy) and third trimester ultrasound findings

Ultrasound examinations were performed at the Fetal Medicine unit during either the second or third trimester in 16 pregnancies. Second trimester ultrasounds (usually between 18-23 weeks gestational age), offer the best opportunity for detailed assessment of fetal anatomy. Fetal anatomy ultrasounds, prior to 24-weeks gestational age, were available for 11/16 pregnancies. Findings from ultrasounds at the Fetal Medicine unit performed during the second and third trimesters are summarized in **Table 5**.

Table 5: Second and third trimester ultrasound findings

Affected Fetuses	F01	F02	F03	F05	F06	F08	F09	F10	F11
LMPS Recurrence	-	-	-	-	-	-	-	-	+
Gestation (weeks)	26.6	19.0	17.5	21.6	18.5	27.5	28.4	19.3	18.3
Live fetus	+	+	-	+	+	+	+	+	+
Fetal movements	reduced	absent	N/A	reduced	absent	absent	reduced	absent	reduced
Presenting part	cephalic	breech	cephalic	cephalic	breech	breech	cephalic	breech	breech
Increased NF	+	NR	+	+	+	NR	+	NR	+
NF (mm)	16.0	NR	NR	15.0	7.7	NR	13.3	NR	7.5
Oedema	+ severe	+ moderate	+ severe	+ mild	+	+ severe	+ moderate	+	+ mild
Hydrops fetalis	+	+	+	-	+	+	-	+	-
Pleural effusion(s)	+	+	+	-	+	+	-	+	-
Pericardial effusion		+	-	-	+	-	-	-	-
Fetal ascites	+	+	+	-	-	-	-	-	-
Cystic hygroma	-	+	+	+	+	-	-	-	-
Abnormal joint flexion	-	+	-	+	-	+	+	-	+
Pterygia	-	-	-	-	+	-	+	+	+
Hands / Fingers	-	-	-	overlapping	-	-	-	clenched	-
Feet	-	talipes	-	rockerbottom	talipes	rockerbottom	-	talipes	talipes
Micrognathia	+	+	-	+	+	-	+	+	+
Chest	-	small	-	pulmonary hypoplasia	pulmonary hypoplasia	-	small	pulmonary hypoplasia	small
Cardiac	mitral regurgitation	-	-	-	-	-	-	-	-
GIT	collapsed stomach	stomach collapsed	-	-	large gallbladder	large gallbladder	-	stomach collapsed	-
Urinary tract	-	-	-	-	-	N	hydronephrosis bilateral	-	megacystis, hydronephrosis bilateral
Scoliosis/ Spine	-	+	-	-	+	neck hyperextended	-	+	-
Soft markers / Other anomalies	-	strawberry sign, increased PT, hypertelorism, short long bones	-	hypoplastic NB	increased PT cardiac EF	brachycephaly, low set ears, short long bones	hypoplastic NB, flat facial profile	hyperechoic bowel	hypoplastic NB
Amniotic fluid	poly	N	oligo	poly	N	poly	poly	N	poly
Cord	cysts	N	N	N	N	cysts, cord oedema	N	N	N
Placenta	N	enlarged	N	N	N	N	small	N	N

Affected Fetuses	F12	F14	F16	F17	F20	F21	F24	F25
LMPS Recurrence	-	-	-		-	-	-	+
Gestation (weeks)	24.3	23.0	21.0		22.6	34.1	21.3	15.2
Live fetus	+	+	+	-	+	+	+	+
Fetal movements	absent	absent	reduced	NA	reduced	normal	normal	absent
Presenting part	breech	cephalic	cephalic	NR	cephalic	cephalic	breech	breech
Increased NF	+	+	+	NR	NR	NR	+	+
NF (mm)	13.0	32.0	NR	NR	NR	NR	12.2	14.0
Oedema	+	+ severe	+ moderate	NR	+ severe	+ severe	+	+ moderate
Hydrops fetalis	+	+	-	NR	+	+	+	-
Pleural effusion(s)	+	+	-	NR	+	+	+	-
Pericardial effusion	+	+	-	NR	-	-	-	-
Fetal ascites	-	+	-	NR	+	-	-	-
Cystic hygroma	-	+	-	NR	-	-	-	-
Abnormal joint flexion	-	-	+	NR	+	-	+	-
Pterygia	+	-	+	NR	+	-	+	-
Hands / Fingers	overlapping	clubbed	overlapping	NR	adducted thumbs	-	overlapping	clubbed
Feet	talipes	pointed	talipes	NR	talipes	-	talipes	talipes
Micrognathia	-	-	+	NR	-	-	+	-
Chest	pulmonary hypoplasia	pulmonary hypoplasia	-	NR	-	pulmonary hypoplasia	pulmonary hypoplasia	-
Cardiac	increased cardiothoracic ratio	ventricular septal defect + coarctation	-	NR	-	-	-	-
GIT	-	-	-	NR	-	-	-	-
Urinary tract	-	-	-	NR	-	dilated bladder hyperechoic renal pelvices	bilateral hydronephrosis	-
Scoliosis/ Spine	neck hyperextended	-	-	NR	-	-	abnormal sacral skin	-
Soft markers / Other anomalies	flat facial profile, cardiac EF	short long bones	hypoplastic NB, increased PT	NR	increased PT, cardiac EF	brachycephaly, mild ventriculomegaly, cardiac EF, hyperechoic bowel	cardiac EF	short long bones, reversed ductus venosus flow
Amniotic fluid	poly	N	N	NR	poly	anhydramnios due to PPRM	N	N
Cord	N	N	N	NR	N	N	N	N
Placenta	N	enlarged	N	NR	enlarged	N	N	N

Table 5 Abbreviations: EF – echogenic focus, N – normal, NA not applicable, NB -nasal bone, NR – not recorded, poly – polyhydramnios, PPRM – preterm prelabour rupture of membranes PT – prenasal thickness,

Gestational age ranged from 15 weeks 2 days to 34 weeks 1 days. Two of these ultrasounds were in women with an LMPS recurrence (F11 and F25). Fifteen fetuses were alive and 2 fetuses (F03 and F17) had no fetal heart activity at index ultrasound. F17, the vanishing affected twin of F16, had a crown rump length compatible with 13 weeks gestational age. Further sonographic assessment of F17 was not completed and is therefore excluded from these prenatal imaging results.

In the 15 live fetuses reduced or absent fetal movements were reported in 13/15 (87%). All fetuses (16/16) had generalised subcutaneous oedema and where reported, an increased nuchal fold thickness (range 7.5 - 32mm); 69% (11/16) met criteria for hydrops fetalis, defined as accumulation of fluid in two or more fetal compartments; 5/16 (31%) had cystic hygromas.

Additional anomalies frequently detected on these ultrasound examinations include: abnormal position of the feet, i.e. talipes or rockerbottom, in 12/16 (75%), pulmonary hypoplasia or a small chest in 10/16 (63%), micrognathia in 9/16 (56%), abnormal joint flexion or positioning in 8/16 (50%), pterygia in 8/16 (50%), abnormal position of the hands or fingers in 8/16 (50%), abnormal curvature of the spine or positioning of the neck in 5/16 (31%). Less frequent findings included: 4/16 fetuses with urinary tract findings, i.e. bilateral hydronephrosis (3/4), megacystis (1/4) and a dilated bladder (1/4), and one fetus with a ventricular septal defect (VSD) and aortic coarctation.

Most fetuses (14/16) had at least one additional soft marker on ultrasound. The following markers were reported most frequently: cardiac echogenic focus (5/14), short long bones (4/14), hypoplastic nasal bone (4/14) and increased prenasal thickness (4/14).

Amniotic fluid volume was normal in 44% (7/16) and increased in 44% (7/16) of pregnancies. Reduced liquor volume (oligohydramnios) was reported for one pregnancy (F03) and another (F21) had anhydramnios due to preterm prelabour rupture of membranes (PPROM). Apart from F21, polyhydramnios was present on all ultrasounds performed after 24 weeks (4/5).

4.6.3 Findings on ultrasounds performed outside the Fetal Medicine unit

Five women had ultrasound examinations at Tygerberg Hospital, but outside the Fetal Medicine unit, i.e. either in labour ward, obstetric admissions or in the obstetric outpatient clinic. When women present with an IUD or if an immediate concern regarding fetal condition exists necessitating urgent intervention or delivery, they are not routinely referred for formal assessment at the Fetal Medicine unit. Results from ultrasounds performed at Tygerberg hospital, but outside the Fetal Medicine unit are reflected in **Table 6**.

At the time of the first ultrasound 3/5 fetuses had already demised. The presence of oedema, hydrops fetalis and cystic hygromas were noted in one demised fetus. In the two living fetuses (F19 and F23) the presence of oedema, hydrops fetalis or reduced fetal movements were not reported, and Caesarean section was performed in both cases due to concern for poor fetal condition based on cardiotocography. The presence of polyhydramnios was documented on three of these informal ultrasounds. There were no additional anomalies reported in any of these ultrasounds.

Table 6: Findings on ultrasounds performed outside the Fetal Medicine unit

Affected Fetuses	F07	F15	F18	F19	F23
Gestation (weeks)	30.0	28.4	25.5	28	27.4
Live fetus	-	-	-	+	+
Fetal movements	NA	NA	NA	NR	NR
Oedema	+ severe	NR	NR	NR	NR
Hydrops fetalis	+	NR	NR	NR	NR
Cystic hygroma	+	NR	NR	NR	NR
Amniotic fluid	poly	poly	NR	NR	poly
Additional reported findings/ anomalies	-	-	-	-	-

Abbreviations Table 6: NA- not applicable, NR- not reported, poly- polyhydramnios

4.7 Phenotypic features on external examination of the fetus

88% (22/25) of fetuses were examined by a member of the medical genetics team following delivery. Three fetuses could not be examined following delivery due to surgical TOP (F04, F25) or TOP being performed at a peripheral hospital (F13). In these three cases the diagnosis of LMPS was certain based on typical prenatal sonographic features of LMPS and confirmed recurrence of LMPS in another pregnancy from the same mother.

The most frequent phenotypic features on external examination of the fetus with LMPS are represented in **Table 7**. Photographs were available to supplement dysmorphology assessments from clinical notes in 32% (7/22) of fetuses.

All fetuses (22/22) had flexion contractures (arthrogryposis) with pterygia of multiple joints in both the upper and lower limbs (**Figure 5** and **Figure 6**). The typical appearance of pterygia involving the neck, axilla, elbow, hips, and knees are demonstrated in **Figure 8**. Generalised subcutaneous pitting oedema was documented in 68% (15/22) of cases. Pitting oedema is demonstrated by an impression mark left on the wrist of F23 in **Figure 8**. Fetal weight for gestational age was below the 10th percentile, therefore small for gestational age, in 50% (11/22) of fetuses.

A short neck 64% (14/22), webbed neck 55% (12/22) or excess nuchal skin 41% (9/22) were frequently reported. Cystic hygromas were present in 18% (4/22) of fetuses and 41% (9/22) of fetuses had palatal anomalies, i.e. high and narrow palate (7/22) or cleft palate (2/22).

Posterior photographs (**Figure 7**) demonstrate scoliosis of the spine and a prominent coccyx present in 36% (8/22) of fetuses. The loss of normal spinal curvatures producing an abnormally straight appearance to the spine is demonstrated on lateral photographs (**Figure 6**). Although the appearance of muscle bulk was not routinely reported in medical records, markedly reduced bulk of the gluteal and deltoid muscles is visible on photo review and is demonstrated in **Figure 7** and **Figure 8**.

Table 7: Clinical phenotypic features and dysmorphism on external fetal examination

Fetus	F01	F02	F03	F05	F06	F07	F08	F09	F10	F11	F12	F14	F15	F16*	F17*	F18	F19	F20*	F21*	F22*	F23*	F24*	T	
Sex	F	F	M	F	M	F	M	F	F	M	M	M	F	M	U	F	F	M	F	U	F	M	O T A L	
Gestational age (w)	27	19	17	23	23	30	27	34	19	20	25	26	28	21	13	25	28	23	34	12	28	22		
Fetal weight (g)	800	142	280	600	570	300	930	1650	NR	350	1000	570	440	450	18	770	1050	541	3700	<50	820	375		
Weight for GA (%)	<3 rd	<3 rd	>97 th	79 th	45 th	<3 rd	12 th	<3 rd	NR	63 rd	>97 th	<3 rd	<3 rd	81 st	<3 rd	56 th	9 th	27 th	>97 th	<3 rd	<3 rd	<3 rd		
Small for gestational age HP:0001518	X	X				X		X				X	X		X		X			X	X	X	11	
General Examination, Musculoskeletal and Soft tissues																								
Flexion contractures of joints HP:0001371	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	22
Multiple Pterygia HP:0001040	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	22
Generalised Oedema HP:0007430			X		X		X		X			X	X	X	X	X	X	X	X	X	X	X	X	15
Short neck HP:0000470		X			X			X	X	X		X	X	X	X			X	X	X	X	X	X	14
Webbed neck HP:0000465				X	X							X		X	X	X	X	X	X	X	X	X	X	12
Camptodactyly HP: 0012385	X						X	X		X		X		X		X	X	X	X		X	X	12	
Talipes equinovarus HP:0001762	X	X			X									X	X		X	X	X	X	X	X	X	11
Narrow shoulders HP:0000774				X								X		X	X	X		X	X	X	X	X	X	10
Excess nuchal skin HP:0000474		X		X										X	X			X	X	X	X	X	X	9
Scoliosis HP:0002650		X												X	X		X	X	X		X	X	X	8
Prominent coccyx HP:0040016		X												X	X			X	X	X	X	X	X	8
Hypoplastic (narrow) pelvis HP:0008839														X	X			X	X	X	X	X	X	7
High, narrow palate HP:0002705	X	X						X						X				X				X	X	7
Absent palmar creases HP:0010489	X	X					X										X	X						5
Cystic hygroma HP:0000476		X	X			X										X								4

Fetus	F01	F02	F03	F05	F06	F07	F08	F09	F10	F11	F12	F14	F15	F16*	F17*	F18	F19	F20*	F21*	F22*	F23*	F24*	TOT
Hypoplastic male external genitalia HP:000050														X				X				X	3
Hypoplastic female external genitalia HP:0012815																			X		X		2
Cleft Palate HP:0000175												X					X						2
Postaxial polydactyly HP:0001162										X												X	2
Facial dysmorphism																							
Low-set ears HP:0000369					X			X	X	X		X		X	X	X	X	X	X	X	X	X	14 (7)*
Micrognathia HP:0000347		X			X		X	X	X	X				X	X		X	X	X	X	X	X	14 (7)*
Hypertelorism HP:0000316		X								X		X		X	X			X	X	X	X	X	10 (7)*
Downslanted palpebral fissures HP:0000494								X		X				X	X		X	X	X		X	X	9 (6)*
Flat face HP:0012368								X		X				X	X		X	X	X		X	X	9 (6)*
Depressed nasal bridge HP:0005280												X		X	X		X	X	X		X	X	8 (6)*
Brachycephaly HP:0000248		X												X	X			X	X	X	X	X	8 (7)*
Anteverted nares HP:0000463										X				X	X			X	X		X	X	7 (6)*
Hypoplastic nasal tip HP:0005278														X	X			X	X		X	X	6 (6)*
Short nose HP:0003196														X	X			X	X		X	X	6 (6)*
Epicanthic folds HP:0000286														X		X		X	X		X	X	6 (5)*
Underdeveloped nasolabial folds HP: 00010801														X				X	X		X	X	5 (5)*
Small mouth HP:0000160														X				X	X		X	X	5 (5)*
Long philtrum HP:000343														X							X	X	3 (3)*

*Fetuses with supplementary assessments from photographs are highlighted in greyscale

Figure 5: Frontal photographs

F16



F23



F24



Figure 6: Lateral photographs

F16 and F17



F23



F24



Figure 7: Posterior photographs

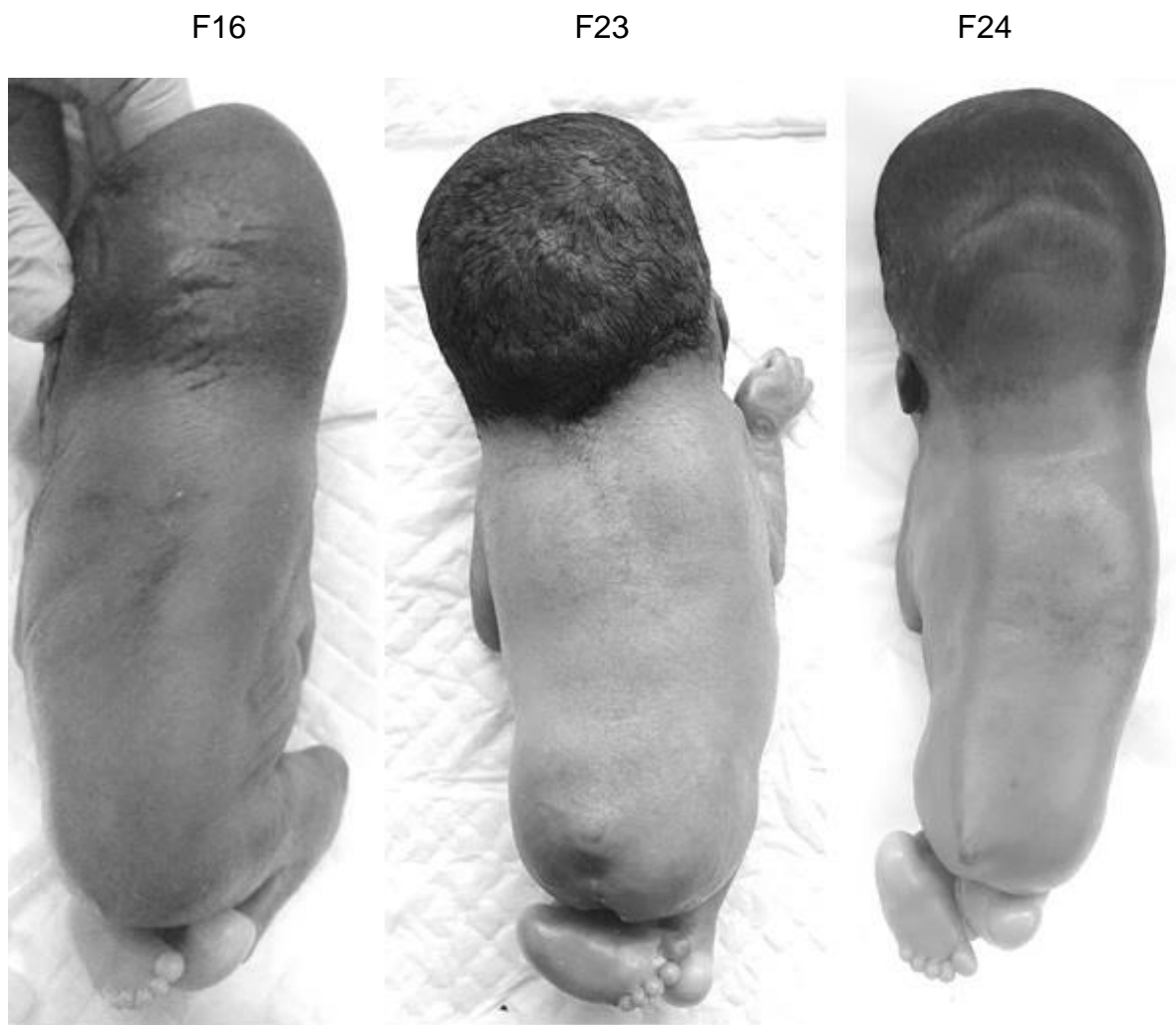


Figure 8: Musculoskeletal anomalies of the upper and lower limbs (F23). Elbow pterygium, upper limb muscle atrophy and subcutaneous pitting oedema on the wrist (left). Axillary pterygium, camptodactyly and webbed neck (middle). Inguinal and popliteal pterygia (right)



Most frequent anomalies of the upper limbs included camptodactyly (12/22), narrow shoulders (10/22), absent palmar creases (5/22) and postaxial polydactyly (2/22), unilateral and bilateral, respectively. In the lower limbs, talipes equinovarus (11/22) and the appearance of a narrow pelvis (7/22) were most frequently reported.

Clinical notes often focused on the gross musculoskeletal anomalies and soft tissue anomalies, neglecting comprehensive documentation of facial dysmorphism. Facial dysmorphism assessments from anterior and lateral photographs were available for 7 fetuses. Typical facial dysmorphism of LMPS is represented in both frontal and lateral photographs of **Figure 5** and **Figure 6**.

Review of facial dysmorphism on anterior and lateral photographs revealed the following: 100% (7/7) of fetuses had low-set ears, micrognathia, hypertelorism, brachycephaly, 86% (6/7) had downslanted palpebral fissures, flat facial profile, depressed nasal bridge, anteverted nares, short nose and hypoplastic nasal tip. Epicanthic folds, underdeveloped nasolabial folds and small mouth were present in 71% (4/7) and a long philtrum in 43% (3/7).

Several characteristic features of LMPS, e.g. fixed flexion of major joints and pterygia are already present and recognizable in a 12-week fetus (F22) (**Figure 9**).

Figure 9: External features of a 12 weeks fetus with LMPS (F22).



4.8 Macroscopic and microscopic findings on autopsy

Results from formal autopsy were available for 10 fetuses and are represented in **Table 8**. Internal examination on F17, the affected vanishing twin of F16, was limited due to small fetal size and extent of autolysis

▪ **Macroscopic examination**

The presence of arthrogryposis and pterygia of all major joints in the upper and lower limbs were confirmed in all fetuses. All 9 fetuses with an internal examination had pulmonary hypoplasia. Additional macroscopic findings on autopsy included: intracranial haemorrhages (3/9), hypoplastic heart (2/9), genitourinary anomalies (2/9) and large bowel malrotation (1/9). Cleft palate detected in two fetuses on external examination, was confirmed on autopsy.

▪ **Microscopic examination**

CNS and muscle histology were often reported as being within normal limits or precluded by autolysis. Muscle histology in F14 and F19, revealed atrophic changes and F16 had variation in muscle fibre size. F06 had brain parenchymal changes suggestive of hypoxic ischemic encephalopathy. Staining of the α -motor neurons were only performed in F19 and appeared normal.

4.9 Radiographic features

Postmortem fetal radiography was available for 6 fetuses and the results are represented in **Table 9**. 50% of fetuses had both AP and lateral images. X-ray quality was generally good, except F02 with a very rotated image. Generalised bone mineral density appeared normal in most (5/6) and reduced in F17, a severely macerated fetus. AP radiographs (**Figure 10**) demonstrate anomalies of the thorax, namely crowded ribs (5/6), abnormally high arched clavicles (5/6) and winged scapula (4/6). Ribs were consistent in number, 12 pairs in all fetuses, but varied notably in their shape i.e. broad in F15 and slender in F16, F17 and especially gracile in F20. Although F20 had normal generalised bone mineralisation, the ribs appeared unusually gracile and under mineralised compared to those of other fetuses.

Table 8: Macroscopic and microscopic findings on autopsy

Fetus	F02	F06	F14	F16	F17	F19	F20	F21	F23	F24
Macroscopic										
Bone and joints	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs	Contractures upper and lower limbs
Soft tissues	Pterygia	Pterygia	Pterygia	Pterygia	Pterygia	Pterygia	Pterygia	Pterygia	Pterygia	Pterygia
Brain	Autolysis	IVH	Autolysis	N	Autolysis	N	IVH	N	N	SDH
Spine	N	N	N	Scoliosis	N	Scoliosis	N	Scoliosis	Scoliosis	Scoliosis
Lungs	Hypoplastic	Hypoplastic	Hypoplastic	Hypoplastic	Autolysis	Hypoplastic	Hypoplastic	Hypoplastic	Hypoplastic	Hypoplastic
Heart	N	N	Hypoplastic Possible VSD	N	Autolysis	N	Hypoplastic	N	N	N
Palate	Intact	Intact	Cleft	Intact	Autolysis	Cleft	Intact	Intact	Intact	Intact
Gastrointestinal	N	Large bowel malrotation	N	N	Autolysis	Congested liver	N	N	N	N
Genitourinary	N	N	Ureters dilated	N	Autolysis	N	N	N	N	Nephromegaly HN
Microscopic										
Brain	Autolysis	IVH HIE	Autolysis	N	Autolysis	N	IVH	N	N	N
Muscle	Autolysis	NR	Atrophy	Variation in muscle fibre size	Autolysis	Patchy atrophic myofibers, Increased endomysial tissue	N	N	N	N
Spinal	NR	NR	NR	NR	NR	N α motor neurons	NR	NR	NR	NR

Abbreviations Table 8: HIE- hypoxic ischaemic encephalopathy, HN – hydronephrosis, IVH -intraventricular haemorrhage, N – normal, NR – not recorded, SDH – subdural haemorrhage

Table 9: Radiographic features on postmortem fetogram

Fetus	F02	F15	F16	F17	F20	F23
Gestation (weeks)	19	28	21	13 (CRL)	23	28
X-ray type	Oblique	AP	AP+ Lateral	Lateral	AP+ Lateral	AP+ Lateral
X-ray quality	Rotated	Adequate	Good	Adequate	Good	Good
Bone Density	Normal	Normal	Normal	Reduced	Normal, except ribs	Normal
Skull	Normal	Normal	Normal	Undermineralised	Normal	Normal
Ribs	Crowded	Broad	Crowded Slender Bell-shaped chest	Crowded Slender Undermineralised	Crowded Gracile Undermineralised	Crowded
Clavicles	High arched	High arched	High arched	Unable to comment	High arched	High arched
Scapulae	Unable to comment	Winged	Winged	Unable to comment	Winged	Winged
Spine	Scoliosis Loss of normal curvature	Scoliosis	Thoracic kyphosis No lumbar lordosis Posterior angulation of sacrum and coccyx	Thoracic kyphosis No lumbar lordosis Posterior angulation of sacrum and coccyx	Thoracic kyphosis No lumbar lordosis Posterior angulation of sacrum and coccyx	Thoracic kyphosis Reduced lumbar lordosis Posterior angulation of sacrum and coccyx
Vertebral fusion	No	No	No	No	No	Possible lumbosacral
Pelvis	Unable to comment	Unable to comment	Hypoplastic Iliia	Unable to comment	Hypoplastic Iliia	Hypoplastic Iliia
Long bones	Straight	Straight	Straight	Straight	Straight	Straight
Fractures	No	No	No	No	No	No
Joints	Flexed, no visible fusions	Flexed, no visible fusions	Flexed, no visible fusions	Flexed, no visible fusions	Flexed, no visible fusions	Flexed, no visible fusions
Soft tissue shadow	Increased, large cervical cystic hygroma	Increased	Increased	Increased	Increased	Increased

Abbreviations Table 9: AP: anteroposterior, CRL – crown rump length

Figure 10: Anteroposterior fetograms

F15



F16



F20



F23



Figure 11: Lateral fetograms

F02 (Oblique)



F16



F17



F20



F23



All fetuses had evidence of an abnormal curvature of the spine. Four fetuses with lateral fetograms (**Figure 11**) had a similar thoracic kyphosis with loss of the normal lumbar lordosis and abnormal posterior angulation of the coccyx resulting in an abnormally straight appearance of the lower thoracic and lumbar region. The AP and lateral fetogram of F23 suggest possible bony vertebral fusions from L3 to the sacrum, but this was not confirmed on postmortem examination.

Due to overlying flexed lower limbs, the pelvis was often difficult to assess. The iliac wings appeared hypoplastic in three fetuses (F16, F20 and F23). All fetuses had straight long bones with no visible fractures. Large joints were in flexion with no bony joint fusions visible. All fetuses had increased soft tissue shadows and F02 had an additional large soft tissue shadow in the neck due to cystic hygroma.

4.10 Placental findings

The placenta appeared normal on most prenatal ultrasounds (15/19), enlarged in three (F02, F14, F20) and small for gestational age in one (F09). Enlarged placentas were always seen with hydropic fetuses, but hydropic fetuses did not always have enlarged placentas. Appearance of the placenta was not documented in 6 pregnancies, either because only informal ultrasonography was performed or due to IUD at time of the ultrasound. Umbilical cord cysts were visible on two ultrasounds (F01, F08) and an SUA in one (F13). Formal placental histology was not available for any case. Macroscopic examination of the placenta by the medical geneticist in F02, confirmed an enlarged placenta with multiple pale areas, suggesting placental oedema.

4.11 Results of standard of care genetic investigations

Chromosome analysis was performed prenatally for three fetuses, i.e. 46, XX (F01), 46, XY (F03), 46, XY (F04). No fetus had aneuploidy or an unbalanced chromosomal profile. Quantitative Fluorescent Polymerase Chain Reaction (QFPCR) in another fetus (F14) also revealed no aneuploidy for chromosomes 21, 18, 13 or the sex chromosomes.

5. Results: Genomic investigation using WES and bioinformatics

WES was performed on an initial trio, mother (M14), father and affected fetus (F16). This trio was selected for genomic investigation due to the availability of both parents to provide informed consent, parental and fetal samples with sufficient DNA quantity and quality for WES, comprehensive prenatal sonography, phenotypic and autopsy information to support a definite diagnosis of LMPS in the fetus, and recurrence of LMPS in more than one pregnancy. The parents have had three fetuses affected with LMPS, i.e. DCDA twins (F16 and F17) and a subsequent affected pregnancy (F22). The fetus (F16) selected for WES was male and the sex of the other two fetuses remained undetermined due to early gestational age. DNA was subsequently also obtained from F22 for confirmation of variant segregation with disease should WES reveal candidate variants from the initial trio.

5.1 Variant annotation and filtering

The trio's exome sequencing data were entered into the bioinformatics pipeline as described in **Section 3.52**. Taper™ and Varseq®, were used to filter variants according to criteria provided in **Figure 2**.

The outcome of variant filtering with Taper™ is shown in **Table 10**. A total of 36 107 variants remained after filtering out polymorphisms, with minor allele frequency (MAF) of >1%, and weakly conserved variants that are highly variable across different species with negative GERP scores. A further 27 608 variants were synonymous or non-frameshift variants unlikely to result in altered protein products. The remaining variants were 8298 missense variants, 77 frameshift deletions, 43 frameshift insertions, 68 stop codon gains (nonsense variants) and 13 stop codon losses (nonstop variants).

Taper™ could not easily filter and compare variants between the proband and unaffected parents. The more user-friendly interface of Varseq® allowed for efficient filtering and comparison of variant segregation between the proband and parents (**Table 11**).

Table 10: Taper TM variant filtering in the proband (F16)

Variant type/filter	Number of variants
Variants remaining after <u>exclusion</u> of variants with: MAF>1% and negative GERP scores	36107
Synonymous and non-frameshift variants	27608
Nonsynonymous SNVs/ missense variants	8298
Frameshift deletion	77
Frameshift insertion	43
Stop gain (Nonsense variant)	68
Stop loss (Nonstop variant)	13

Table 11: Varseq [®] variant filtering in the proband (F16)

Variant type/filter		Number of remaining variants
De novo heterozygous variants (not inherited from either parent)		89
Homozygous variants with heterozygous segregation in parents	Total	56
	Missense	52
	In-frame deletions/insertions	3
	5'UTR premature start gain	1
	Stop gain / stop loss / splice site / frameshift	0
Compound heterozygous variants with heterozygous segregation in parents	Total	218
	Missense	211
	In-frame deletions/insertions	3
	Frameshift	1
	Splice site	2
Stop gain / stop loss	1	
Chromosome X hemizygous variants (not hemizygous in father and not homozygous in mother)		3

5.2 Identification, interpretation, and classification of variants in genes of interest

Following the process of variant identification in the proband (**Section 3.5.2**), a list of candidate variants (**Table 12**) was compiled for further interpretation and classification, prior to verification with Sanger sequencing. No clearly pathogenic or disease-causing variants were identified.

5.2.1 Variants in known LMPS disease genes

No variants of interest were detected in genes encoding components of the AChR and neuromuscular junction typically associated with LMPS, i.e. *CHRNA1*, *CHRND*, *CHRNA3*, *CHRNA4*, *CHRNA5*, *CHRNA7*, *CHRNA9*, *CHRNB1*, *CHRNB2*, *CHRNB3*, *CHRNB4*, *CHRNB5*, *CHRNB6*, *CHRNB7*, *CHRNB8*, *CHRNB9*, *CHRNB10*, *CHRNB11*, *CHRNB12*, *CHRNB13*, *CHRNB14*, *CHRNB15*, *CHRNB16*, *CHRNB17*, *CHRNB18*, *CHRNB19*, *CHRNB20*, *CHRNB21*, *CHRNB22*, *CHRNB23*, *CHRNB24*, *CHRNB25*, *CHRNB26*, *CHRNB27*, *CHRNB28*, *CHRNB29*, *CHRNB30*, *CHRNB31*, *CHRNB32*, *CHRNB33*, *CHRNB34*, *CHRNB35*, *CHRNB36*, *CHRNB37*, *CHRNB38*, *CHRNB39*, *CHRNB40*, *CHRNB41*, *CHRNB42*, *CHRNB43*, *CHRNB44*, *CHRNB45*, *CHRNB46*, *CHRNB47*, *CHRNB48*, *CHRNB49*, *CHRNB50*, *CHRNB51*, *CHRNB52*, *CHRNB53*, *CHRNB54*, *CHRNB55*, *CHRNB56*, *CHRNB57*, *CHRNB58*, *CHRNB59*, *CHRNB60*, *CHRNB61*, *CHRNB62*, *CHRNB63*, *CHRNB64*, *CHRNB65*, *CHRNB66*, *CHRNB67*, *CHRNB68*, *CHRNB69*, *CHRNB70*, *CHRNB71*, *CHRNB72*, *CHRNB73*, *CHRNB74*, *CHRNB75*, *CHRNB76*, *CHRNB77*, *CHRNB78*, *CHRNB79*, *CHRNB80*, *CHRNB81*, *CHRNB82*, *CHRNB83*, *CHRNB84*, *CHRNB85*, *CHRNB86*, *CHRNB87*, *CHRNB88*, *CHRNB89*, *CHRNB90*, *CHRNB91*, *CHRNB92*, *CHRNB93*, *CHRNB94*, *CHRNB95*, *CHRNB96*, *CHRNB97*, *CHRNB98*, *CHRNB99*, *CHRNB100*, *RAPSN*, *RAPSN2*, *RAPSN3*, *RAPSN4*, *RAPSN5*, *RAPSN6*, *RAPSN7*, *RAPSN8*, *RAPSN9*, *RAPSN10*, *RAPSN11*, *RAPSN12*, *RAPSN13*, *RAPSN14*, *RAPSN15*, *RAPSN16*, *RAPSN17*, *RAPSN18*, *RAPSN19*, *RAPSN20*, *RAPSN21*, *RAPSN22*, *RAPSN23*, *RAPSN24*, *RAPSN25*, *RAPSN26*, *RAPSN27*, *RAPSN28*, *RAPSN29*, *RAPSN30*, *RAPSN31*, *RAPSN32*, *RAPSN33*, *RAPSN34*, *RAPSN35*, *RAPSN36*, *RAPSN37*, *RAPSN38*, *RAPSN39*, *RAPSN40*, *RAPSN41*, *RAPSN42*, *RAPSN43*, *RAPSN44*, *RAPSN45*, *RAPSN46*, *RAPSN47*, *RAPSN48*, *RAPSN49*, *RAPSN50*, *RAPSN51*, *RAPSN52*, *RAPSN53*, *RAPSN54*, *RAPSN55*, *RAPSN56*, *RAPSN57*, *RAPSN58*, *RAPSN59*, *RAPSN60*, *RAPSN61*, *RAPSN62*, *RAPSN63*, *RAPSN64*, *RAPSN65*, *RAPSN66*, *RAPSN67*, *RAPSN68*, *RAPSN69*, *RAPSN70*, *RAPSN71*, *RAPSN72*, *RAPSN73*, *RAPSN74*, *RAPSN75*, *RAPSN76*, *RAPSN77*, *RAPSN78*, *RAPSN79*, *RAPSN80*, *RAPSN81*, *RAPSN82*, *RAPSN83*, *RAPSN84*, *RAPSN85*, *RAPSN86*, *RAPSN87*, *RAPSN88*, *RAPSN89*, *RAPSN90*, *RAPSN91*, *RAPSN92*, *RAPSN93*, *RAPSN94*, *RAPSN95*, *RAPSN96*, *RAPSN97*, *RAPSN98*, *RAPSN99*, *RAPSN100*, *DOK7*, *MUSK*.

A single maternally inherited heterozygous missense variant was detected in *NEB*. Similarly, a single paternally inherited heterozygous missense variant was detected in *RYR1*. Given the autosomal recessive pattern of inheritance associated with the LMPS phenotype, the presence of isolated heterozygous variants in *NEB* and *RYR1* is insufficient to result in an LMPS phenotype. Furthermore, the variant in *NEB* has a ClinVar entry (ID: 331514) supporting a classification of likely benign or benign, whereas the classification of the variant in *RYR1* remains uncertain (ID: 478217). Further Sanger verification and classification of these two variants were not pursued since neither are expected to be sufficient to contribute to disease.

5.2.2. Variants in genes associated with broader fetal akinesia phenotypes

Four heterozygous missense variants were detected in four different genes associated with fetal akinesia and neuromuscular phenotypes, i.e. *SYNE1*, *DMPK*, *TTN*, *KLHL41*. Similar to the variants in *RYR1* and *NEB*, the variants in *SYNE1*, *DMPK*, *TTN* and *KLHL41*, did not segregate in a manner sufficient to explain the phenotype in the proband. All variants were maternally inherited, except the variant in *SYNE1* which is de novo and classified as benign according to ACMG-AMP criteria.

Table 12: Candidate variants interpretation and classification

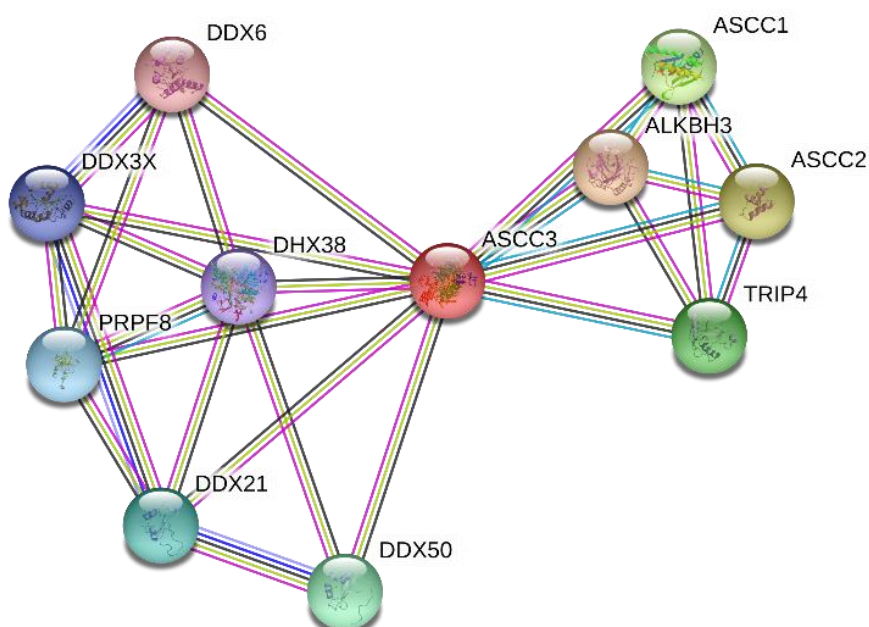
Gene	Variant coordinates	SNP identifier	Amino acid change	Variant type	Zygoty proband	Zygoty parents	Population frequency (gnomAD)	Conservation (GERP score)	In silico predictions: FATHMM SIFT CADD	ClinVar	ACMG-AMP classification	Known Disease/Phenotype and inheritance pattern
Variants in known LMPS disease genes												
<i>NEB</i>	NM_001271208.1:c.3826C>A	rs34234609	p.Pro1276Thr	missense	het	het (mat)	0.00242 1/413	5.466	Tolerated Damaging 22.7	Benign/ Likely Benign	Likely Benign	AR- nemaline myopathy AR- LMPS
<i>RYR1</i>	NM_000540.2:c.2680C>A	rs111976711	p.Pro894Thr	missense	het	het (pat)	0.00000406 1/246418	4	Damaging Tolerated 15.79	Uncertain	VUS	AD- malignant hyperthermia AR- FADS, LMPS, myopathy
Variants in known fetal akinesia spectrum disease genes												
<i>SYNE1</i>	NM_033071.3:c.24743G>A	rs148008634	p.Arg8248Gln	missense	het	de novo	0.000313 1/3199	5.34	Tolerated Tolerated 14.15	Conflicting	Benign	AD- Emery Dreifuss muscular dystrophy AR- AMC, spinocerebellar ataxia 8
<i>DMPK</i>	NM_001288764.1:c.971C>G	rs149803658	p.Ser324Cys	missense	het	het (mat)	0.000877 1/1139	4.48	Tolerated Damaging 26.4	No entry	VUS	AD - Myotonic dystrophy type 1
<i>TTN</i>	NM_001267550.2:c.32254G>A	rs72650028	p.Val10752Ile	missense	het	het (mat)	0.00281 1/356	4.94	Tolerated Tolerated 21.4	Benign/Likely Benign	Benign	AD- cardiomyopathy, myopathy, muscle dystrophy AR- limb girdle muscle dystrophy, myopathy
<i>KLHL41</i>	NM_006063.2:c.913C>T	rs149971244	p.Leu305Phe	missense	het	het (mat)	0.000223 1/4490	5.32	Tolerated Damaging 22.5	Likely benign	Likely benign	AR- nemaline myopathy
Variants in gene pathways interacting with known LMPS or fetal akinesia spectrum disease genes												
<i>ASCC3</i>	NM_006828.3:c.299C>T	rs551963299	p.Thr100Ile	missense	hmz	het (mat) het (pat)	0.00000801 1/124873	5.84	Tolerated Tolerated 18.51	No entry	NA - VUS	None
Variants of interest in genes not known to be associated with LMPS, fetal akinesia or their known pathways												
<i>SCYL1</i>	NM_020680.3:c.899G>A	rs373266891	p.Ser300Asn	missense	hmz	het (mat) het (pat)	0.00000802 1/124705	5.57	Tolerated Tolerated 18.46	No entry	Likely Benign	AR- spinocerebellar ataxia 21
<i>IGHMBP2</i>	NM_002180.2:c.581C>T	rs375694606	p.Thr194Ile	missense	compound het	het (mat)	0.00000795 1/125710	4.92	Damaging Tolerated 15.75	No entry	VUS	AR- Charcot Marie Tooth type 2S neuropathy
<i>IGHMBP2</i>	NM_002180.2:c.2545G>A	rs2228208	p.Ala849Thr	missense	compound het	het (pat)	0.000377 1/2654	4.07	Damaging Tolerated 9.37	Likely benign	Likely benign	

Abbreviations Table 12: AD – autosomal dominant, AMC – arthrogyrosis multiplex congenita, AR – autosomal recessive, FADS – fetal akinesia deformation sequence, het- heterozygous, hmz – homozygous, LMPS – lethal multiple pterygium syndrome mat – maternal, NA- not applicable, pat – paternal, VUS- variant of uncertain significance

5.2.3 Variants in gene pathways interacting with LMP5 and fetal akinesia genes

Using STRINGdb, genes interacting with known LMP5 and fetal akinesia genes were identified. This approach identified a homozygous missense variant of interest in *ASCC3*, which segregated heterozygous in each parent. *ASCC3* currently has no known disease association but interacts with other subunits of the Activating Signal co-integrator 1 (ASC-1) complex, i.e. *ASCC1*, *ASCC2* and *TRIP4* (**Figure 12**). The role of *ASCC1* and *TRIP4* in normal neuromuscular developmental has been established (Knierim *et al.*, 2016; Böhm *et al.*, 2019; Villar-Quiles *et al.*, 2020). The *ASCC3* variant is discussed further as a candidate variant in a gene of interest in **Section 5.3**.

Figure 12: The ASC-1 pathway with *ASCC3* and interactor genes, including *ASCC1* and *TRIP4*. Image from STRINGdb (Szklarczyk *et al.*, 2019)



5.2.4 Variants in genes or gene pathways not known to interact with LMPS and fetal akinesia genes or their pathways

During variant filtering, several variants of interest were identified in genes with no known interaction with known LMPS or fetal akinesia genes. These variants were of interest either due to the variant type, predicted effect on protein function, population frequency, gene function or associated phenotype. Several variants with loss of function effects, e.g. nonsense, splice site, frameshift (**Table 10** and **Table 11**) were assessed, however none of these variants were homozygous or compound heterozygous with each other. None of the genes with these loss of function variants had a known association with fetal akinesia or LMPS. Further evaluation of these variants deemed pathogenicity unlikely.

5.3 Further variant interpretation and classification of candidate variant(s) in gene(s) of interest

No clearly pathogenic or disease-causing variants were identified. The homozygous missense variant in *ASCC3*, NM_006828.3:c.299C>T was of interest due to the gene's shared pathway with other genes, i.e. *ASCC1* and *TRIP4*, associated with a fetal akinesia phenotype. The presence of this variant was confirmed with Sanger sequencing. The variant was assessed for pathogenicity using several standardised tools:

5.3.1. Population data

The population database, gnomAD (Karczewski *et al.*, 2020), reports only two heterozygotes with this variant. Both heterozygotes are of African descent. No homozygotes have been reported in any population. This translates to an extremely low population allele frequency of 0.00000801 or $1/124\,873$.

5.3.2 Evolutionary conservation data

The reference amino acid, threonine, is highly conserved at that position with a GERP score of 5.899 (reference range -12.3 to 6.17; 6.17 being the most conserved) (Davydov *et al.*, 2010).

5.3.3 Computational data and predictions

This missense variant results in a threonine to isoleucine amino acid change at position 100. The effect of this amino acid change was assessed with HOPE (Venselaar *et al.*, 2010). The mutant residue, isoleucine, is bigger and more hydrophobic than the wild type threonine. The variant therefore introduces a more hydrophobic residue, which can result in loss of hydrogen bonds or disturb correct protein folding. UniProt (Venselaar *et al.*, 2010) indicates that the variant is located within a stretch of residues required for interaction with *ASCC2*. Although these differences in amino acid properties may disturb this region and its function, in silico predictions with FATHMM and SIFT show that this amino acid change is likely to be tolerated. This variant has a CADD score of 18.51, which predicts that the amino acid substitution falls between the top 1% to 10% of most deleterious variants across the genome (Rentzsch *et al.*, 2019).

5.3.4 Functional data

No functional data for this variant is available.

5.3.5 Variant segregation data

The proband's parents are both heterozygotes for the variant. Due to technical difficulties with primer binding in the laboratory, segregation of the variant in the proband's affected sib, F22, or other affected fetuses could not be assessed.

5.3.6 ACMG/AMP variant classification

The ACMG/AMP variant classification tool (**Figure 3**) is intended for classification of variants in known morbid genes. Since *ASCC3* currently has no proven disease association i.e. it is a gene of uncertain significance, these guidelines should not be applied for variant classification.

However, should a disease association for *ASCC3* be established, two variant pathogenicity rules, PM2 and BP1, would currently be met. The very low population frequency and absence of homozygotes with this variant supports pathogenicity (PM2). However, the absence of other missense variants in *ASCC3* being known disease-causing, would support a benign nature of this variant (BP1).

Table 13: ACMG/AMP variant classification for *ASCC3* c.299C>T homozygous

ACMG/AMP Rule	Pathogenicity	Explanation
PM2	Pathogenic moderate	gnomAD allele count is less than threshold
BP1	Benign supporting	18/18 (100%) of previously reported non-VUS missense variants in <i>ASCC3</i> are benign
ACMG/AMP classification: Variant of Uncertain Significance		

In summary, this variant is currently classified as a variant of uncertain significance in a gene of uncertain significance.

5.4 Incidental findings

The parents of the proband indicated during the consenting process that they wish to have incidental findings reported. No known pathogenic or likely pathogenic variants were detected in any of the 59 actionable genes recommended by the ACMG.

6. Discussion

6.1 LMPS prevalence in our population

The index case series of 25 fetuses with LMPS is the first description of LMPS in Southern Africa and to the best of our knowledge the largest from a single institution internationally. Over an 8-year period we diagnosed an average of two to three LMPS cases per year.

Compared to City of Cape Town demographics, there appears to be a disproportionate number of women of Black African ancestry with fetuses affected with LMPS, 90% in our study versus 38.6% (Census 2011). Ethnicity was not well documented, though when specified, parents were almost exclusively Xhosa. Since consanguinity is not a cultural norm among black South Africans, it is not surprising that no couples reported relatedness.

65% of women in our cases series had no comorbidities. The remaining 35% were well-controlled on chronic medication. Their comorbidities and chronic medications are not expected to cause LMPS. Women did not have a personal or family history of neuromuscular disorders. Although our case series included two twin pregnancies, a risk factor for intrauterine constraint and secondary fetal deformation, the extent of the LMPS phenotype is considered too severe.

Similar to previous reports (Ravenscroft *et al.*, 2011; Abdalla *et al.*, 2017; Beecroft *et al.*, 2018), our data strongly support an intrinsic, genetic aetiology of LMPS. LMPS is for the most part considered an autosomal recessive genetic condition with a handful of case reports reporting apparent X-linked recessive types (Meyer-Cohen J, Dillon A, Pai GS, 1999; Tolmie *et al.*, 2018). A similar male to female sex distribution (40% males, 44% females, 16% undetermined) among affected fetuses, recurrence of LMPS in sibships with the same paternity (20%) and an absence of a broader multigenerational family history of either LMPS or other neuromuscular disease in all, lends support to a possible autosomal recessive pattern of inheritance in our population.

Several examples of autosomal recessive conditions due to homozygous pathogenic variants exist in the Black South African population, e.g. oculocutaneous albinism type 2, Fanconi Anaemia, Cystic Fibrosis and Bardet-Biedl syndrome (Krause, Seymour and Ramsay, 2018). When both alleles in an autosomal recessive condition share the same haplotype background, a founder effect or genetic drift is likely (Tan-Sindhunata *et al.*, 2015). Autosomal recessive founder mutations have been established for several international populations with lethal fetal akinesia phenotypes (Pakkasjärvi *et al.*, 2006; Tan-Sindhunata *et al.*, 2015).

Due to our small sample size and variation in LMPS numbers per year, an estimation of LMPS prevalence in our population is approached with caution. Considering a birth prevalence (live and stillbirths >500g) of 40,000- 50,000 per year within our referral area, the estimated birth prevalence of LMPS could be as high as 1 per 20,000. Although this appears much higher than current international estimates (Orphanet, 2020), LMPS might not be as rare as historically reported. A recent review (McPherson, 2019) of hydrops fetalis in second trimester miscarriages and stillbirths reports an LMPS phenotype in 6% (17/277) of cases. Due to intrauterine lethality in either the second or third trimester, LMPS is probably under recognized and its prevalence therefore underestimated in both local and international populations.

The apparent increased frequency of LMPS in our population, especially in Black South Africans, and a probable autosomal recessive pattern of inheritance may suggest genetic drift or a founder effect, although our data are currently insufficient to support this theory. Given the genetic heterogeneity of LMPS, more than one genetic cause and inheritance pattern of LMPS is possible. Historical under recognition of LMPS may contribute to a perceived increased frequency of LMPS in our population.

6.2 LMPS pregnancy outcomes and complications

In keeping with known second trimester or third trimester lethality, no pregnancy in our study continued beyond 34 weeks and no fetus survived peripartum resuscitative

efforts. Overall pregnancy outcomes were 56% TOP, 36% spontaneous IUD or stillbirth and 2% ENND.

Prenatal recognition of non-viability or an antenatal diagnosis of LMPS was possible in 72% of cases. In view of inevitable lethality, it is not surprising that 78% of women opted for TOP following an antenatal diagnosis of LMPS. Early recognition during the first trimester, has the additional benefit of allowing maternal choice regarding TOP method, i.e. either surgical or medical.

22% (4/18) of pregnancies were ongoing following antenatal diagnosis of LMPS. These pregnancies were not without maternal risks, with half of mothers (2/4) experiencing complications related to continued pregnancy, i.e. preeclampsia and severe hydrops fetalis precluding vaginal delivery (1/4), and ASHPT with severe polyhydramnios and preterm labour (1/4) in a twin pregnancy discordant for LMPS. These two women with hypertensive related complications at 34 weeks gestation may have had mirror syndrome related to fetal or placental hydrops.

Fetal non-viability or LMPS were not recognized antenatally in 28 % (7/25) of cases. None of these women had formal antenatal sonography and either presented with an IUD of unknown cause (5/7) or in preterm labour with concern regarding fetal condition on cardiotocography (2/7) prompting urgent intervention. The most significant complication associated with delayed or failed recognition of fetal non-viability were Caesarean sections for fetal indications (2/7).

Our research indicates that risks to maternal health in ongoing pregnancies are not negligible and warrant discussion during prenatal counselling. Consideration of maternal risks may inform parental pregnancy decision making and management. In ongoing pregnancies, antenatal diagnosis allows for monitoring for maternal complications e.g. mirror syndrome, polyhydramnios and delivery planning which should aim to reduce Caesarean section for fetal indications and resuscitation of non-viable fetuses.

6.3 Prenatal detection and ultrasound findings of LMPS

76% of fetuses had formal antenatal sonography of which 12% were NT ultrasounds, 58% second trimester (prior to 24 weeks) and 26% after 24 weeks. These results agree with the existing evidence that features of LMPS can be diagnosed as early as first trimester, but that most cases are not diagnosed until the 2nd or 3rd trimester (Hall, 2009).

First trimester NT sonography had a 100% (3/3) detection rate of features indicating severe fetal akinesia and secondary fetal deformation i.e. reduced or absent fetal movements, increased NT with generalised oedema and abnormal flexion of all major joints. NT measurements were increased in all fetuses with LMPS but varied widely from mild to severe (range 3.9 - 12.88mm). For 2/3 cases, NT ultrasounds were done in the context of LMPS recurrence, therefore sonologists were specifically looking for features of LMPS. Pterygia may already be visible on 1st trimester antenatal ultrasound (Chen, 2012; Filges and Hall, 2013) but were not reported in any of our 1st trimester ultrasounds despite often being done in the context of a possible LMPS recurrence.

Generalised oedema was consistently detected with formal sonography in 100% of fetuses and in every trimester. Fetuses frequently met criteria for hydrops fetalis which appears to increase with pregnancy progression, from 66% on NT scan to 80% >24 weeks. In view of these results and McPherson et al.'s report (McPherson, 2019) of an LMPS phenotype in 6% of their cohort with hydrops fetalis, sonographic review of fetuses with non-immune hydrops fetalis, especially when recurrent, should include an assessment for features suggestive of LMPS.

Generalised oedema and/or fetal hydrops is a characteristic feature of LMPS which distinguishes the condition from other syndromes or phenotypes associated with fetal akinesia, e.g. FADS (Moerman *et al.*, 1990). Several mechanisms for fetal hydrops in LMPS have been proposed. Jugular lymphatic obstruction sequence due to delayed development of jugular lymphatics and subsequent delayed drainage of lymph into the internal jugular vein, is a commonly accepted theory (Moerman and Fryns, 1990; Moerman *et al.*, 1990). This sequence is not specific to LMPS and is observed in the

pathogenesis of hydrops in chromosomal anomalies, e.g. Turner syndrome, and single gene disorders, e.g. Noonan syndrome. Lymph vessels and muscles are both mesodermal structures, therefore an early intrinsic or extrinsic insult to these tissues could provide an alternative explanation for the co-occurrence of fetal akinesia and hydrops (Moerman *et al.*, 1990). Although other types of fetal akinesia are not generally associated with hydrops, it is plausible that absent fetal movement likely exacerbates lymphoedema due to an inability to generate pressure in deep lymphatics to facilitate adequate venous return (Moerman *et al.*, 1990). At least some LMPS affected fetuses (Froster *et al.*, 1997), including two from our case series, have evidence of deficient cardiac muscle. Progressive cardiac failure due to deficient cardiac muscle activity should be explored as an alternative mechanism of pathogenesis.

Apart from generalised oedema and hydrops fetalis, the most frequent findings on second and third trimester sonography included positional deformities of the feet (75%), pulmonary hypoplasia (63%), micrognathia (56%), abnormal fixed flexion of major joints and fingers (50%) and visible pterygia of major joints (50%). An unexpected finding was that 20% of fetuses (5/25) had evidence of dilation of the genitourinary tract on sonography, i.e. bilateral hydronephrosis (2/5), megacystis (1/5), megacystis and bilateral hydronephrosis (1/5), and dilated bladder with hyperechoic renal pelvises (1/5). These findings are not commonly associated with LMPS, but have been infrequently reported in both LMPS and FADS (de Die-Smulders, Schranders-Stumpel and Fryns, 1990; Hall, 2009). These anomalies could either represent an underlying obstructive uropathy, e.g. posterior urethral valves, which may be unrelated to LMPS, or represent pseudo-obstruction due to genitourinary smooth muscle dysfunction similar to megacystis-microcolon hypoperistalsis syndrome (Fontanella *et al.*, 2019). Further macroscopic and microscopic investigation of the genitourinary tract is warranted to delineate the underlying pathogenesis associated with these findings.

Due to decreased fetal swallowing, polyhydramnios is expected to develop in all pregnancies with LMPS continuing beyond the first trimester (Hall, 2014). Polyhydramnios was reported on formal sonography in 4/5 pregnancies continuing beyond 24 week and either contributed to or caused PPRM resulting in anhydramnios in the other pregnancy. Polyhydramnios was not consistently observed

prior to this gestation (earliest gestation 18+3 weeks). Apart from preterm labour and amniotic fluid drainage in one DCDA pregnancy discordant for LMPS, maternal complications due to polyhydramnios were few. This is perhaps, at least in part due to 83% of pregnancies not progressing beyond the second trimester.

In all sixteen cases where sonography was performed in the Fetal Medicine unit, LMPS or fetal non-viability was recognized antenatally. This suggests that a delayed or missed diagnosis of LMPS at our institution is presumably not due to an inability to recognise LMPS on formal sonography but rather due to local circumstances or protocols, e.g. a delayed time until first antenatal visit and limited availability of 1st trimester NT sonography. In our setting, NT sonography is limited to tertiary institutions for women with clear indications. Since LMPS is detectable on first trimester ultrasound, we recommend NT sonography in all women with a previous affected pregnancy. Early recognition of LMPS during the 1st or early 2nd trimester, offers the option of offering surgical TOP, which can be performed as an outpatient procedure.

In our case series, all women with a known LMPS recurrence presented for sonography in a subsequent pregnancy, half of these (2/4) were NT scans. Prenatal recognition of LMPS accompanied by genetic counselling regarding autosomal recessive inheritance and recurrence risk empower women to make informed decisions regarding their current and future pregnancy management.

6.4 LMPS dysmorphism on external examination

External examination of affected fetuses confirmed the presence of multiple fixed flexion deformities of upper and lower limbs and pterygia of upper and lower limbs joints in all fetuses (22/22). Although prenatal ultrasound consistently reported presence of oedema or hydrops fetalis, the presence of subcutaneous oedema was only documented in 68% of examinations. This may in part be due to fetal maceration at the time of examination or incomplete clinical documentation. Fetal weight for gestational age varied greatly, i.e. small for gestational age (50%), average for gestational age (32%), large for gestational age (14%) and not reported (4%). All fetuses with weights >97th centile were hydroptic, but not all hydroptic fetuses were

large for gestational age. The presence of hydrops fetalis alone does not explain the variation in weight for gestational age.

Lower reported frequency of features on clinical and dysmorphology examination is likely due to description of these features in free text as opposed to using a research orientated checklist during data collection. This is especially true for fetuses whose records were retrospectively reviewed and where staff changes occurred. The evaluation of photos proved to be a valuable adjunct in the dysmorphology examination and demonstrated a recurrent pattern of facial dysmorphology.

Review of photographs of seven affected fetuses, revealed a consistent pattern of facial dysmorphology. Bearing in mind that two fetuses were only 12 and 13 weeks gestational age, all the following features were present in five or more fetuses: hypertelorism, downslanted palpebral features, epicanthic folds, depressed nasal bridge, short nose, anteverted nares with a hypoplastic nasal tip, underdeveloped nasolabial folds, a small mouth, micrognathia, flat facial profile, low set ears and brachycephaly. These facial features are similar to those previously reported by Froster et al. in their review of several LMPS case series (Froster *et al.*, 1997). Most of these features probably relate to the jugular venous obstruction sequence, as overlap with phenotypic features seen in Turner and Noonan syndrome is apparent. Other features, e.g. underdeveloped nasolabial folds, small mouth and micrognathia, may be explained by lack of facial and perioral muscle movements, jaw activity and swallowing. Palatal anomalies were reported in 40% of our cases, which are less frequent than the 75% prevalence previously reported (Froster *et al.*, 1997).

The presence of postaxial polydactyly type B, not previously reported with LMPS, is expected to be unrelated to LMPS and rather explained by the increased birth prevalence (approximately 1%) of postaxial polydactyly in Black Africans (Kromberg and Jenkins, 1982).

6.5 Value of Autopsy in LMPS

Postmortem examinations were performed in 40% (10/25) of fetuses and confirmed classical findings of LMPS in all fetuses, i.e. multiple joint contractures, pterygia, and pulmonary hypoplasia. Autopsy was useful in identifying anomalies of internal organs that would have remained undetected on external examination alone, i.e. the presence of hypoplastic hearts (2/10), genitourinary anomalies (2/10) and large bowel malrotation (1/10).

Three fetuses had evidence of either intraventricular or subdural haemorrhages. Although early and severe CNS pathology can result in an LMPS phenotype (Spearritt, Tannenbergh and Payton, 2018), all three fetuses had normal formal antenatal ultrasounds with no report of antenatal intracranial pathology. These haemorrhages are therefore not thought to be the cause of LMPS in these cases, but probably the result of prematurity and birth related trauma.

Hypoplasia of the heart, defined as a small heart with a reduced weight for gestational age, has previously been reported in LMPS but not uniformly (Froster *et al.*, 1997). One fetus (F14) with a hypoplastic heart on postmortem had a VSD and aortic coarctation documented on antenatal ultrasound. Only the VSD was subsequently demonstrated on autopsy. F14 had atrophic skeletal muscle on histology which may suggest an underlying cause of LMPS affecting both skeletal and cardiac muscle though cardiac muscle histology was not available. The other fetus (F20) with a hypoplastic heart, had no prenatal evidence of an associated cardiac anomaly or skeletal muscle atrophy on histology. Both fetuses had severe hydrops fetalis with enlarged placentas.

Due to inevitable intrauterine lethality, fetuses are often macerated at delivery with a variable degree of autolysis. The presence of autolysis precluding adequate microscopic examination of the brain, spine and muscles is a problem also faced by other researchers investigating LMPS neuromuscular pathology (Cox *et al.*, 2003). Muscle pathology in our case series agrees with the histological diversity reported by Cox *et al.* in their review of LMPS in 14 fetuses (Cox *et al.*, 2003). Similar to Cox *et al.*,

we also report cases with increased variation in muscle fibre size (F16), simple atrophy (F14 and F19) and an increase in interstitial connective tissue or endomysial tissue (F19). We did not demonstrate overt muscle dystrophy seen in primary muscular dystrophies, or vacuolar myopathic changes seen in glycogen storage disease (Cox *et al.*, 2003).

In contrast to Cox *et al.*'s results, where only 1/14 fetuses had normal muscle histology, the majority (4/7) of fetuses in our case series had normal histology. A possible explanation may be the use of special stains and immunohistochemistry by Cox *et al.*, which are not readily or routinely available in our setting. Immunohistochemistry of components of the neuromuscular junction, especially the AChR, and skeletal muscle may provide valuable aetiological clues thereby facilitating more targeted genetic testing.

6.6 Radiographic phenotype of LMPS

Postmortem radiography was performed in 24% of fetuses. Similar to previous case series (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990; Moerman *et al.*, 1990; Froster *et al.*, 1997; Cox *et al.*, 2003), radiographic features in our study were not uniform. Several features were however consistent in all fetuses (6/6), i.e. an abnormal curvature of the spine, flexion of major joints without visible bony joint fusions, straight long bones without fractures and increased soft tissue shadows due to subcutaneous oedema.

All four fetuses with lateral X-rays had a similar loss of lumbar and sacral lordosis with posterior angulation of the coccyx resulting in an unusually straight appearance of the lumbosacral region and subcutaneous bony prominence of the coccyx. Although spinal scoliosis is reported often but not consistently in LMPS (Froster *et al.*, 1997), the characteristic curvature seen in our case series has not been described in similar detail previously.

It is unclear whether these features represent secondary deformation due to fetal akinesia or a direct result of vertebral or cartilaginous fusions. Among fetuses with

lateral radiography, the only fetus with possible vertebral fusions was also of most advanced gestation at 28 weeks. Cartilaginous vertebral or joint fusions at earlier gestations would not be visible on X-ray. Specialised postmortem staining with alizarian red or blue may identify cartilaginous fusions (Froster *et al.*, 1997; Cox *et al.*, 2003), however this was not available in any of our cases.

Decreased bone mineralisation and fractures were not a hallmark feature in our case series. Reduced bone mineralisation in F17 is likely attributable to gross maceration at the time of radiography. Hall (Hall, 2014) suggests that osteoporosis and perinatal fractures in fetal akinesia result from reduced mechanical stress on long bones, as opposed to a primary defect in bone modelling. A correlation between more advanced gestations and osteopenia could not be demonstrated in our case series, although radiography was not available for fetuses with gestations beyond 28 weeks.

Ribs appeared notably crowded in the majority of fetuses (5/6) which is attributable to pulmonary hypoplasia. The presence of either broad or slender ribs, is consistent with previous case reports. (Froster *et al.*, 1997; Cox *et al.*, 2003). Tolmie *et al.* (Tolmie *et al.*, 2018) suggested an X-linked recessive pattern of inheritance associated with broad ribs. However, the only fetus with broad ribs in our case series was female, making an X-linked cause in our case unlikely.

6.7 Placental findings in LMPS

Placental oedema appears to be the most frequent placental finding in LMPS (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990; Moerman *et al.*, 1990; Froster *et al.*, 1997). In our case series, all placentas appeared normal on 1st trimester NT sonography (3/3). Most placentas, 75% (12/16), continued to appear normal on 2nd and 3rd trimester sonography, 3/16 were enlarged and 1/16 small for gestational age. Since placentas are not routinely examined by either clinical genetics or anatomical pathology, placental morphology and histology was not well documented in our case series which may have resulted in an underreporting of placental oedema. A small placenta in F09 is unexpected and more likely due to maternal hypertensive related complications.

6.8 Standard of care genetic investigations

LMPS is a single gene disorder most often caused by pathogenic sequence variants (Ravenscroft *et al.*, 2011; Beecroft *et al.*, 2018). Standard chromosome analysis or aneuploidy QFPCR would therefore not detect LMPS and were not routinely performed in our case series. As anticipated and similar to previous reports (de Die-Smulders, Schrandt-Stumpel and Fryns, 1990; Cox *et al.*, 2003) all four fetuses with standard chromosomal investigations had normal results. Chromosome analysis or chromosomal microarray may have a role in cases where features are atypical, or where the cause for fetal hydrops is uncertain, but molecular genetic or genomic investigations would be more appropriate where a clinical diagnosis of LMPS is certain. A targeted LMPS gene panel could be useful in clinical practice, but a greater understanding of the underlying genetic aetiologies of LMPS in our population would be important to ensure its analytic and clinical validity.

6.9 Genomic investigation of LMPS using WES

Initial trio WES and bioinformatics analysis did not detect any variants of interest in known LMPS or fetal akinesia genes. Up to 27% of unrelated individuals with severe fetal akinesia will remain without a genetic diagnosis despite deep clinical phenotyping combined with WES and CNV analysis (Pergande *et al.*, 2020). Several reasons for failure to detect disease-causing variants in our proband are considered:

- WES is targeted to sequencing of protein coding regions, therefore pathogenic variants in introns and regulatory regions such as promoters or enhancers will be missed.
- It is possible, though unlikely, that exonic disease-causing sequence variants were inadvertently filtered out during our bioinformatic workflow. Due to limited DNA from the proband and additional cost implications, separate CNV analysis could not be performed.
- Although adequate sequencing depth can potentially detect copy number variation, our bioinformatic workflow is not optimised for this purpose.

- The recent surge in novel genes and pathways associated with LMPS and historical underrepresentation of African populations in genomic research, support the possibility that the cause of LMPS may be unique in our population.

Several candidate variants were considered and evaluated for their potential to cause LMPS using standardised tools. Although none of these variants could be linked to LMPS, several characteristics of the variant in Activating signal co-integrator 1 complex subunit 3 (*ASCC3*) are of interest but remain insufficient to prove its role in fetal akinesia or LMPS.

6.10 The role of *ASCC3* and its pathway in disease

ASCC3 is located on chromosome 6q16.3 and functions as a 3'-5' DNA helicase which promotes DNA unwinding and repair of alkylated DNA. It is the largest subunit of the highly conserved tetrameric ASC-1 complex (**Figure 12**). ASC-1 is ribonuclear protein complex participating in transcriptional activation and RNA processing events as a novel cell cycle regulator (Knierim *et al.*, 2016; Villar-Quiles *et al.*, 2020).

Although *ASCC3* does not currently have a specific disease association or phenotype, its role in resistance of alkylation damage in tumour cell lines was previously established (Soll *et al.*, 2018). *ASCC3* interacts with other subunits of the ASC-1 complex, i.e. *ASCC1*, *ASCC2* and *TRIP4* (Knierim *et al.*, 2016; Villar-Quiles *et al.*, 2020). Importantly, *ASCC1* appears to interact with the ASC-1 complex through the *ASCC3* subunit (Soll *et al.*, 2018).

The role of *ASCC1* and *TRIP4* in normal prenatal neuromuscular development has been established. Functional studies by Knierim *et al.* (Knierim *et al.*, 2016) identified high expression levels of *ASCC1* and *TRIP4* in the spinal cord, brain, paraspinal ganglia, thyroid, and submandibular glands of 17 day old mouse embryos. Knockout of *TRIP4* and *ASCC1* in zebrafish, disrupt alpha motor neuron outgrowth, myotome and neuromuscular junction formation resulting in swimming defects (Knierim *et al.*, 2016). Biallelic loss of function (frameshift and nonsense) variants in *ASCC1* and *TRIP4* cause a lethal myopathy, spinal muscular atrophy and congenital bone

fractures type 2 (SMABF2) (Knierim *et al.*, 2016; Böhm *et al.*, 2019). SMABF2 is a severe autosomal recessive neuromuscular disorder characterised by prenatal onset of hypokinesia resulting in AMC and a propensity for prenatal long bone fractures. Early lethality during the neonatal period commonly occurs due to respiratory insufficiency and feeding difficulties (Knierim *et al.*, 2016; Böhm *et al.*, 2019). An association between the ASC-1 complex and other neuromuscular phenotypes, e.g. amyotrophic lateral sclerosis and cardiomyopathy has since been established (Chi *et al.*, 2018; Villar-Quiles *et al.*, 2020).

The variant in *ASCC3* has a very low allele frequency with no homozygotes observed in any population. The amino acid appears conserved with a physicochemical difference between the wild-type and mutant. Amino acid substitutions caused by missense mutations, may influence protein folding and binding. Although computational predictions did not predict pathogenicity of this variant, they are not always representative of what occurs *in vivo*.

The role of the ASC-1 complex in disease is only beginning to be understood. Further research may elucidate the effect of variants in *ASCC3* on other subunits of the ASC-1 complex and its role in disease over time. Demonstrating segregation of this homozygous variant with LMPS in other affected fetuses would add supporting evidence to a possible role in LMPS warranting further functional work.

6.11 Study strengths and limitations

6.11.1 Strengths

This is the first comprehensive description of LMPS in the South African population. A variety of data sources were available for identification of LMPS cases. To limit selection bias, retrospective records were meticulously reviewed and cross referenced to ensure only cases meeting the case definition of LMPS are included. Phenotypic data included comprehensive prenatal ultrasonography in most cases, which not only

adds valuable insight into LMPS in early fetal life but has demonstrated that early detection of LMPS is possible in our clinical setting.

6.11.2 Limitations

As a case series conducted at a single institution, the findings may not be generalizable to the broader population of Southern Africa. The selection and description of retrospective cases were dependant on how comprehensively phenotypic information was documented in free text. Both retrospective and prospective cases of LMPS were assessed by different members of the medical genetics and fetal medicine team, therefore differences in the description and reporting of features could have occurred. These differences were mitigated by having photos, X-ray's and autopsy data on a significant number of cases.

Due to the lethal nature of LMPS, autolysis of fetal tissue proved challenging during microscopic postmortem assessments. Electron microscopy, immunohistochemistry of receptors at the neuromuscular junction and staining of skeletal muscle components could be a useful guide towards more targeted molecular genetic testing. These special stains are not routinely available but useful to obtain should further investigation of this research cohort be pursued.

Due to budget and time constraints, WES was only performed in one trio. The raw genomic data of the trio were not available for review after VCF files were generated. Due to technical difficulties in the lab related to primer binding and further delays and restrictions during the COVID-19 epidemic, the candidate variant in *ASCC3* could not be assessed for segregation with other affected fetuses.

7. Conclusion and future research directions

LMPS is a genetically heterogenous and phenotypically distinct expression of severe and early onset of fetal akinesia. LMPS can be clinically differentiated from other fetal akinesia phenotypes and pterygium syndromes. Severe joint contractures involving the upper and lower limbs, extensive skin pterygia and inevitable intrauterine or peripartum lethality (frequently associated with generalised oedema, progressive hydrops fetalis and pulmonary hypoplasia) are characteristic of the LMPS phenotype.

We report the first case series of LMPS in Southern Africa and largest from a single institution internationally. Paucity of LMPS prevalence data and small size of existing LMPS cohorts, suggest extreme rarity of the condition. A cautious estimated prevalence of approximately 1 in 20,000 in our case series appears up to 50-fold higher than previous international estimates. Although an apparent increased LMPS prevalence in the context of an autosomal recessive condition and in a specific population raises the possibility of genetic drift or founder effect, this hypothesis could not yet be supported by our genomic data. A recent study demonstrated a 6% incidence of an LMPS phenotype in hydropic stillbirths, which suggests that LMPS may not be as rare as previously assumed. A high index of suspicion for LMPS should be maintained in pregnancies presenting with non-immune hydrops fetalis, especially if recurrent and with the same partner.

Features of severe fetal akinesia and non-viability are reliably detected from first trimester sonography. Although features of non-viability were always detected on ultrasounds performed at our Fetal Medicine unit, these features were frequently missed by less experienced clinicians in less controlled environments. Maternal risks due to delayed or non-detection of LMPS are not negligible and may result in avoidable Caesarean sections for fetal indications. Early detection of LMPS in pregnancy allows for improved counselling regarding maternal health risks, recurrence risks and pregnancy management options. For these reasons NT sonography should be offered to all women with a previous pregnancy affected with LMPS.

We described and discussed LMPS features using a ‘deep phenotyping’ approach from a variety of sources, i.e. prenatal ultrasounds, external and dysmorphology assessments supplemented by photographs, postmortems (macro-and microscopic), radiographs, placental and standard chromosome analysis results. Several recurrent patterns emerged, especially typical facial dysmorphology, similar radiographic features of the spine and unexpected frequent dilatation along the genitourinary tract. Evidence of possible cardiac and smooth muscle (genitourinary or GI) involvement in some cases, warrant thorough macro- and microscopic assessment for involvement of these tissues in future. The phenotypic diversity of neuromuscular pathology in LMPS may reflect underlying genetic heterogeneity within our cohort or variability in phenotypic expression of the same condition. Acquisition of specialised stains for immunohistochemistry of components of the neuromuscular junction, specifically the AChR, and skeletal muscle may be useful in guiding more targeted genetic testing in future.

As evidenced by the number of recent novel fetal akinesia gene discoveries, knowledge regarding the aetiologic and genetic heterogeneity of fetal akinesia and LMPS is rapidly expanding. Initial genomic investigation with WES in a family with recurrent LMPS in three fetuses did not identify disease-causing variants in known LMPS or fetal akinesia genes. We identified *ASCC3* as a possible gene of interest in LMPS due to its interaction with other subunits of the ASC-1 complex that are associated with a known fetal akinesia phenotype. Knowledge regarding the function of *ASCC3* and its role within the ASC-1 complex and in LMPS may become evident over time. Revisiting our existing genomic data in a few years with improved software and knowledge of novel genes causing fetal akinesia could provide new insights.

As a next step, establishing whether the homozygous missense variant in *ASCC3* segregates with LMPS in other affected fetuses is recommended. Segregation of the variant with LMPS may warrant further transcriptomic or functional research in animal models to demonstrate the effect of this variant in vivo. Considering the possibility of genetic drift or founder effect and an autosomal recessive pattern of inheritance of LMPS in our population, haplotype analysis or homozygosity mapping could be a useful approach to identify autosomal recessive genes identical-by-descent. Alternatively, a broader genomic analysis using WGS in a trio or quad, i.e. both parents

and two affected fetuses or an affected fetus and unaffected child, would provide the benefit of better genomic coverage than WES and increased ability to detect larger deletions or copy number variation.

Collaboration and sharing of knowledge with colleagues from other hospitals across South Africa may provide a better understanding of the prevalence, similarities, and differences of LMPS in the broader South African population. Further genomic investigations, or more targeted genetic investigations guided by neuromuscular immunohistochemistry, could clarify the genetic determinants of LMPS in our population which may be unique.

8. References

- Abdalla, E. *et al.* (2017) 'Lethal multiple pterygium syndrome: A severe phenotype associated with a novel mutation in the nebulin gene', *Neuromuscular Disorders*. Elsevier B.V., 27(6), pp. 537–541. doi: 10.1016/j.nmd.2017.01.013.
- Beecroft, S. J. *et al.* (2018) 'Genetics of neuromuscular fetal akinesia in the genomics era', *Journal of Medical Genetics*, 55(8), pp. 505–514. doi: 10.1136/jmedgenet-2018-105266.
- Böhm, J. *et al.* (2019) 'Novel ASCC1 mutations causing prenatal-onset muscle weakness with arthrogryposis and congenital bone fractures', *Journal of Medical Genetics*, 56(9), pp. 617–621. doi: 10.1136/jmedgenet-2018-105390.
- Chen, C. P. (2012) 'Prenatal diagnosis and genetic analysis of fetal akinesia deformation sequence and multiple pterygium syndrome associated with neuromuscular junction disorders: A review', *Taiwanese Journal of Obstetrics and Gynecology*. Elsevier Taiwan LLC, 51(1), pp. 12–17. doi: 10.1016/j.tjog.2012.01.004.
- Chi, B. *et al.* (2018) 'The neurodegenerative diseases ALS and SMA are linked at the molecular level via the ASC-1 complex', *Nucleic Acids Research*. Oxford University Press, 46(22), pp. 11939–11951. doi: 10.1093/nar/gky1093.
- Cox, P. M. *et al.* (2003) 'Diversity of neuromuscular pathology in lethal multiple pterygium syndrome', *Pediatric and Developmental Pathology*, 6(1), pp. 59–68. doi: 10.1007/s10024-002-0042-9.
- Davydov, E. V. *et al.* (2010) 'Identifying a High Fraction of the Human Genome to be under Selective Constraint Using GERP++', *PLoS Computational Biology*. Edited by W. W. Wasserman. Public Library of Science, 6(12), p. e1001025. doi: 10.1371/journal.pcbi.1001025.
- DeMyer, W. and Baird, I. (1969) 'Mortality and skeletal malformations from amniocentesis and oligohydramnios in rats: Cleft palate, clubfoot, microstomia, and adactyly', *Teratology*. Teratology, 2(1), pp. 33–37. doi: 10.1002/tera.1420020105.
- de Die-Smulders, C. E., Schrandt-Stumpel, C. T. and Fryns, J. P. (1990) 'The lethal multiple pterygium syndrome: a nosological approach', *Genetic counseling (Geneva, Switzerland)*. Department of Clinical Genetics, University Hospital of Maastricht, State University of Limburg, The Netherlands., 1(1), pp. 13–23.
- Drachman, D. B. and Sokoloff, L. (1966) 'The role of movement in embryonic joint development', *Developmental Biology*. Academic Press, 14(3), pp. 401–420. doi: 10.1016/0012-1606(66)90022-4.
- Ellard, S. *et al.* (2015) 'An exome sequencing strategy to diagnose lethal autosomal recessive disorders', *European Journal of Human Genetics*. Nature Publishing Group, 23(3), pp. 401–404. doi: 10.1038/ejhg.2014.120.
- Filges, I. and Hall, J. G. (2013) 'Failure to identify antenatal multiple congenital contractures and fetal akinesia - proposal of guidelines to improve diagnosis', *Prenatal Diagnosis*. Prenat Diagn, 33(1), pp. 61–74. doi: 10.1002/pd.4011.
- Fontanella, F. *et al.* (2019) 'Fetal megacystis: a lot more than LUTO', *Ultrasound in*

Obstetrics and Gynecology. John Wiley and Sons Ltd, 53(6), pp. 779–787. doi: 10.1002/uog.19182.

Froster, U. G. *et al.* (1997) 'Lethal multiple pterygium syndrome: Suggestion for a consistent pathological workup and review of reported cases', *American Journal of Medical Genetics*. John Wiley & Sons, Ltd, 68(1), pp. 82–85. doi: 10.1002/(SICI)1096-8628(19970110)68:1<82::AID-AJMG16>3.0.CO;2-K.

Glanzmann, B. *et al.* (2016) 'A new tool for prioritization of sequence variants from whole exome sequencing data', *Source Code for Biology and Medicine*. BioMed Central Ltd., 11(1). doi: 10.1186/s13029-016-0056-8.

Groza, T. *et al.* (2015) 'The Human Phenotype Ontology: Semantic Unification of Common and Rare Disease', *American Journal of Human Genetics*. Cell Press, 97(1), pp. 111–124. doi: 10.1016/j.ajhg.2015.05.020.

Gundogan, M. *et al.* (2006) 'First Trimester Ultrasound Diagnosis of Lethal Multiple Pterygium Syndrome', *Fetal Diagnosis and Therapy*, 21(5), pp. 466–470. doi: 10.1159/000093890.

Gymrek, M. *et al.* (2013) 'Identifying personal genomes by surname inference'. American Association for the Advancement of Science, 339(6117), pp. 321–324. doi: 10.1126/science.1229566.

Hall, J. G. (1984) 'The lethal multiple pterygium syndromes', *American Journal of Medical Genetics*. Wiley-Blackwell, 17(4), pp. 803–807. doi: 10.1002/ajmg.1320170410.

Hall, J. G. (2009) 'Pena-Shokeir phenotype (fetal akinesia deformation sequence) revisited', *Birth Defects Research Part A - Clinical and Molecular Teratology*. John Wiley & Sons, Ltd, 85(8), pp. 677–694. doi: 10.1002/bdra.20611.

Hall, J. G. (2014) 'Arthrogryposis (multiple congenital contractures): Diagnostic approach to etiology, classification, genetics, and general principles', *European Journal of Medical Genetics*. Elsevier Masson SAS, 57(8), pp. 464–472. doi: 10.1016/j.ejmg.2014.03.008.

Hall, J. G. *et al.* (2018) 'Limb pterygium syndromes: A review and report of eleven patients', *American Journal of Medical Genetics*. Wiley-Blackwell, 12(4), pp. 377–409. doi: 10.1002/ajmg.1320120404.

Hall, J. G. and Kiefer, J. (2016) 'Arthrogryposis as a Syndrome: Gene Ontology Analysis', *Molecular Syndromology*. S. Karger AG, pp. 101–109. doi: 10.1159/000446617.

Hall, J. G., Kimber, E. and Dieterich, K. (2019) 'Classification of arthrogryposis', *American Journal of Medical Genetics, Part C: Seminars in Medical Genetics*, 181(3), pp. 300–303. doi: 10.1002/ajmg.c.31716.

Hartwig, N. G. *et al.* (2018) 'Case of lethal multiple pterygium syndrome with special reference to the origin of pterygia', *American Journal of Medical Genetics*. Wiley-Blackwell, 33(4), pp. 537–541. doi: 10.1002/ajmg.1320330425.

Kalia, S. S. *et al.* (2017) 'Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): A policy statement of the American College of Medical Genetics and Genomics', *Genetics in*

- Medicine*. Nature Publishing Group, 19(2), pp. 249–255. doi: 10.1038/gim.2016.190.
- Karczewski, K. J. *et al.* (2020) 'The mutational constraint spectrum quantified from variation in 141,456 humans', *Nature*. Nature Research, 581(7809), pp. 434–443. doi: 10.1038/s41586-020-2308-7.
- Kariminejad, A. *et al.* (2016) 'Lethal multiple pterygium syndrome, the extreme end of the RYR1 spectrum Pathophysiology of musculoskeletal disorders', *BMC Musculoskeletal Disorders*. BMC Musculoskeletal Disorders, 17(1), pp. 1–5. doi: 10.1186/s12891-016-0947-5.
- Kiefer, J. and Hall, J. G. (2019) 'Gene ontology analysis of arthrogyrosis (multiple congenital contractures)', *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*. Blackwell Publishing Inc., 181(3), pp. 310–326. doi: 10.1002/ajmg.c.31733.
- Knierim, E. *et al.* (2016) 'Mutations in Subunits of the Activating Signal Cointegrator 1 Complex Are Associated with Prenatal Spinal Muscular Atrophy and Congenital Bone Fractures', *American Journal of Human Genetics*. Cell Press, 98(3), pp. 473–489. doi: 10.1016/j.ajhg.2016.01.006.
- Köhler, S. *et al.* (2019) 'Expansion of the Human Phenotype Ontology (HPO) knowledge base and resources', *Nucleic Acids Research*, 47(D1), pp. D1018–D1027. doi: 10.1093/nar/gky1105.
- Krause, A., Seymour, H. and Ramsay, M. (2018) 'Common and Founder Mutations for Monogenic Traits in Sub-Saharan African Populations', *Annual Review of Genomics and Human Genetics*. Annual Reviews, 19(1), pp. 149–175. doi: 10.1146/annurev-genom-083117-021256.
- Kromberg, J. G. R. and Jenkins, T. (1982) 'Common birth defects in South African Blacks', *South African Medical Journal*, 62(17), pp. 599–602.
- Li, H. *et al.* (2009) 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics*, 25(16), pp. 2078–2079. doi: 10.1093/bioinformatics/btp352.
- Li, H. and Durbin, R. (2009) 'Fast and accurate short read alignment with Burrows-Wheeler transform', *Bioinformatics*, 25(14), pp. 1754–1760. doi: 10.1093/bioinformatics/btp324.
- Lowry, R. B. *et al.* (2010) 'Prevalence of multiple congenital contractures including arthrogyrosis multiplex congenita in Alberta, Canada, and a strategy for classification and coding', *Birth Defects Research Part A - Clinical and Molecular Teratology*. Birth Defects Res A Clin Mol Teratol, 88(12), pp. 1057–1061. doi: 10.1002/bdra.20738.
- Lüchinger, A. B. *et al.* (2008) 'Fetal onset of general movements', *Pediatric Research*. Nature Publishing Group, 63(2), pp. 191–195. doi: 10.1203/PDR.0b013e31815ed03e.
- McKenna, A. *et al.* (2010) 'The genome analysis toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data', *Genome Research*. Cold Spring Harbor Laboratory Press, 20(9), pp. 1297–1303. doi: 10.1101/gr.107524.110.
- McKie, A. B. *et al.* (2014) 'Germline mutations in RYR1 are associated with foetal akinesia deformation sequence/lethal multiple pterygium syndrome', *Acta Neuropathologica Communications*. BioMed Central Ltd., 2(1), pp. 1–11. doi: 10.1186/s40478-014-0148-0.

- McPherson, E. (2019) 'Hydrops fetalis in a cohort of 3,137 stillbirths and second trimester miscarriages', *American Journal of Medical Genetics, Part A*, 179(12), pp. 2338–2342. doi: 10.1002/ajmg.a.61340.
- Meyer-Cohen J, Dillon A, Pai GS, Co. S. (1999) 'Lethal Multiple Pterygium syndrome in four male fetuses in a family: evidence for X-linked recessive subtype?', *American journal of medical genetics*, 82(1), pp. 97–99.
- Michalk, A. *et al.* (2008) 'Acetylcholine Receptor Pathway Mutations Explain Various Fetal Akinesia Deformation Sequence Disorders', *American Journal of Human Genetics*, 82(2), pp. 464–476. doi: 10.1016/j.ajhg.2007.11.006.
- Missias, A. C. *et al.* (1996) 'Maturation of the acetylcholine receptor in skeletal muscle: Regulation of the AChR γ -to- ϵ switch', *Developmental Biology*. Academic Press Inc., 179(1), pp. 223–238. doi: 10.1006/dbio.1996.0253.
- Moerman, P. *et al.* (1990) 'Pathogenesis of the lethal multiple pterygium syndrome', *American Journal of Medical Genetics*, 35(3), pp. 415–421. doi: 10.1002/ajmg.1320350319.
- Moerman, P. and Fryns, J. P. (1990) 'The fetal akinesia deformation sequence. A fetopathological approach', *Genetic counseling (Geneva, Switzerland)*. Department of Pathology I, University of Leuven, Belgium., 1(1), pp. 25–33.
- Moessinger, A. C. (1983) 'Fetal Akinesia Deformation Sequence: An Animal Model', *Pediatrics*, 72(6).
- Morgan, N. V. *et al.* (2006) 'Mutations in the Embryonal Subunit of the Acetylcholine Receptor (CHRNG) Cause Lethal and Escobar Variants of Multiple Pterygium Syndrome', *The American Journal of Human Genetics*, 79(2), pp. 390–395. doi: 10.1086/506256.
- Nayak, S. S. *et al.* (2014) 'Fetal akinesia deformation sequence: Expanding the phenotypic spectrum', *American Journal of Medical Genetics, Part A*, 164(10), pp. 2643–2648. doi: 10.1002/ajmg.a.36673.
- Niles, K. M. *et al.* (2019) 'Fetal arthrogryposis multiplex congenita/fetal akinesia deformation sequence (FADS)—Aetiology, diagnosis, and management', *Prenatal Diagnosis*. John Wiley and Sons Ltd, 39(9), pp. 720–731. doi: 10.1002/pd.5505.
- Orphanet (2020) 'Prevalence and incidence of rare diseases : Bibliographic data.', *Orphanet Report Series: Rare Diseases collection*, (1), pp. 1–78.
- Pakkasjärvi, N. *et al.* (2006) 'Lethal congenital contracture syndrome (LCCS) and other lethal arthrogryposes in Finland - An epidemiological study', *American Journal of Medical Genetics, Part A*. Am J Med Genet A, 140(17), pp. 1834–1839. doi: 10.1002/ajmg.a.31381.
- Parashar, S. Y. *et al.* (2006) 'Spectrum of features in pterygium syndrome', *Asian Journal of Surgery*. Asian Surgical Association, 29(2), pp. 104–108. doi: 10.1016/S1015-9584(09)60118-4.
- Pergande, M. *et al.* (2020) 'The genomic and clinical landscape of fetal akinesia', *Genetics in Medicine*. Springer US, 22(3), pp. 511–523. doi: 10.1038/s41436-019-0680-1.

- Quinlan-Jones, E. *et al.* (2019) 'Molecular autopsy by trio exome sequencing (ES) and postmortem examination in fetuses and neonates with prenatally identified structural anomalies', *Genetics in Medicine*. Nature Publishing Group, 21(5), pp. 1065–1073. doi: 10.1038/s41436-018-0298-8.
- Ravenscroft, G. *et al.* (2011) 'Fetal akinesia: Review of the genetics of the neuromuscular causes', *Journal of Medical Genetics*, 48(12), pp. 793–801. doi: 10.1136/jmedgenet-2011-100211.
- Rentzsch, P. *et al.* (2019) 'CADD: Predicting the deleteriousness of variants throughout the human genome', *Nucleic Acids Research*. Oxford University Press, 47(D1), pp. D886–D894. doi: 10.1093/nar/gky1016.
- Richards, S. *et al.* (2015) 'Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology', *Genetics in Medicine*. Nature Publishing Group, 17(5), pp. 405–424. doi: 10.1038/gim.2015.30.
- Rink, B. D. (2011) 'Arthrogyrosis: A review and approach to prenatal diagnosis', *Obstetrical and Gynecological Survey*. Obstet Gynecol Surv, pp. 369–377. doi: 10.1097/OGX.0b013e31822bf5bb.
- Robinson, P. N. *et al.* (2008) 'The Human Phenotype Ontology: A Tool for Annotating and Analyzing Human Hereditary Disease', *The American Journal of Human Genetics*. Elsevier BV, 83(5), pp. 610–615. doi: 10.1016/j.ajhg.2008.09.017.
- Robinson, P. N. (2012) 'Deep phenotyping for precision medicine', *Human Mutation*. Hum Mutat, pp. 777–780. doi: 10.1002/humu.22080.
- Shamseldin, H. E., Swaid, A. and Alkuraya, F. S. (2013) 'Lifting the lid on unborn lethal Mendelian phenotypes through exome sequencing', *Genetics in Medicine*. Nature Publishing Group, 15(4), pp. 307–309. doi: 10.1038/gim.2012.130.
- Shihab, H. A. *et al.* (2014) 'Ranking non-synonymous single nucleotide polymorphisms based on disease concepts', *Human Genomics*. BioMed Central Ltd., 8(1). doi: 10.1186/1479-7364-8-11.
- Sim, N. L. *et al.* (2012) 'SIFT web server: Predicting effects of amino acid substitutions on proteins', *Nucleic Acids Research*. Oxford Academic, 40(W1), pp. W452–W457. doi: 10.1093/nar/gks539.
- Soll, J. M. *et al.* (2018) 'RNA ligase-like domain in activating signal cointegrator 1 complex subunit 1 (ASCC1) regulates ASCC complex function during alkylation damage', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 293(35), pp. 13524–13533. doi: 10.1074/jbc.RA117.000114.
- Spearritt, D. J., Tannenberg, A. E. G. and Payton, D. J. (2018) 'Lethal multiple pterygium syndrome: Report of a case with neurological anomalies', *American Journal of Medical Genetics*. Wiley-Blackwell, 47(1), pp. 45–49. doi: 10.1002/ajmg.1320470110.
- Swinyard, C. A. (1982) 'Concepts of multiple congenital contractures (arthrogyrosis) in man and animals', *Teratology*. John Wiley & Sons, Ltd, 25(2), pp. 247–258. doi: 10.1002/tera.1420250214.
- Szklarczyk, D. *et al.* (2019) 'STRING v11: Protein-protein association networks with

increased coverage, supporting functional discovery in genome-wide experimental datasets', *Nucleic Acids Research*. Oxford University Press, 47(D1), pp. D607–D613. doi: 10.1093/nar/gky1131.

Tan-Sindhunata, M. B. *et al.* (2015) 'Identification of a Dutch founder mutation in MUSK causing fetal akinesia deformation sequence', *European Journal of Human Genetics*. Nature Publishing Group, 23(9), pp. 1151–1157. doi: 10.1038/ejhg.2014.273.

Todd, E. J. *et al.* (2015) 'Next generation sequencing in a large cohort of patients presenting with neuromuscular disease before or at birth', *Orphanet Journal of Rare Diseases*. BioMed Central Ltd., 10(1), p. 148. doi: 10.1186/s13023-015-0364-0.

Tolmie, J. L. *et al.* (2018) 'A lethal multiple pterygium syndrome with apparent X-linked recessive inheritance', *American Journal of Medical Genetics*. Wiley-Blackwell, 27(4), pp. 913–919. doi: 10.1002/ajmg.1320270418.

Venselaar, H. *et al.* (2010) 'Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces', *BMC Bioinformatics*. BMC Bioinformatics, 11. doi: 10.1186/1471-2105-11-548.

Villar-Quiles, R. N. *et al.* (2020) 'ASC-1 Is a Cell Cycle Regulator Associated with Severe and Mild Forms of Myopathy', *Annals of Neurology*. John Wiley and Sons Inc., 87(2), pp. 217–232. doi: 10.1002/ana.25660.

Vogt, J. *et al.* (2008) 'Mutation Analysis of CHRNA1, CHRNB1, CHRND, and RAPSN Genes in Multiple Pterygium Syndrome/Fetal Akinesia Patients', *American Journal of Human Genetics*. Am J Hum Genet, 82(1), pp. 222–227. doi: 10.1016/j.ajhg.2007.09.016.

Vogt, J. *et al.* (2009) 'Germline mutation in DOK7 associated with fetal akinesia deformation sequence', *Journal of Medical Genetics*, 46(5), pp. 338–340. doi: 10.1136/jmg.2008.065425.

Vogt, J. *et al.* (2012) 'CHRNA1 genotype-phenotype correlations in the multiple pterygium syndromes', *Journal of Medical Genetics*, 49(1), pp. 21–26. doi: 10.1136/jmedgenet-2011-100378.

Weiner, C. (2014) 'Anticipate and communicate: Ethical management of incidental and secondary findings in the clinical, research, and direct-to-consumer contexts (December 2013 Report of the Presidential Commission for the Study of Bioethical Issues)', *American Journal of Epidemiology*, 180(6), pp. 562–564. doi: 10.1093/aje/kwu217.

Yehia, L. and Eng, C. (2019) 'Largescale population genomics versus deep phenotyping: Brute force or elegant pragmatism towards precision medicine', *npj Genomic Medicine*. Nature Publishing Group, pp. 1–2. doi: 10.1038/s41525-019-0080-0.

9. Appendix A: Perinatal Losses Data Sheet

PERINATAL LOSSES DATA SHEET (Continued)

DOD: Paediatric Summary:

.....

Clinical Examination by GENETICIST: Date of Examination:.....
 Astraia:.....
 Results of Antenatal Genetic Testing:.....
 Examination: Male / Female / Ambiguous Condition: Fresh / Macerated / In Rigor

Est. Gestation:.....w Weight:.....g FL:.....mm HC:.....mm
 Notes:.....

Possible Diagnosis / Syndrome:.....
 Post Mortem Requested: YES / NO
 Other Investigations:.....
 Recommendations:.....

Geneticist Name: Signature:
COMBINED ADVICE FOR NEXT PREGNANCY and PLANNED FOLLOW-UP:

- Interpregnancy Interval ofyrs recommended / Pregnancy NOT recommended
- Pregestational Folate / HPT control / Glycaemic control
- Early pregnancy ASPRIN / Calcium / Progesterone / Stop Statin
- Glucose Profile Recommended atw
- Antenatal Level of Care: MOU / Level I / Level II / Level III
- Genetic Counselling & NT / Early Detail Scan ~ 11-13w
- Cervical Screening atw
- History Indicated Cervical Cerclage / Transabdominal Cerclage
- Umbilical Artery Doppler Screen 24w
- Other dopplers (MCA, Ut Art, DV) at gestation.....w
- Growth monitoring to include growth scans fromw
- Preterm Admission to be offered atw
- Offer Preterm Delivery atw
- Life-Style Modification: Lose Weight / Stop Smoking / Stop Drugs / Stop ETOH

Recall for Post-Natal / Preconceptional Review & Counselling:
 Date:..... HRC / Special Care / Other

Tests to be done:.....

TYGERBERG HOSPITAL

Department of Obstetrics & Gynaecology: General Specialist Services

PERINATAL LOSSES DATA SHEET

Contact Number:
 Name:
 Folder Number:
 DOB:

Stillbirth
 NND

Age: G: / P: / M: / T: / E: Booked: YES / NO
 BMI: Syphilis: POS / NEG Rhesus: POS / NEG HIV: POS / NEG

CD4: VL: HAART / PMTCT / None Date started:.....

Habits in pregnancy: Smoking / Alcohol / Drugs (Methamphetamine/Cannabis/Heroin/Other)

Gestation: w Dates / EUS / LUS / SFH Date delivered:.....

Delivery Method: NVD / Ventouse / Forceps / Breech / Caesarean Section (LUSTI / Classical)

Fetal condition on Admission: Alive / Dead / Unknown At Birth: Alive / FSB / MSB

Birthweight:	Baby A	Baby B
Gender:	M / F	M / F
Apgars:	/ /	/ /

Relevant Previous Obstetric Hx:

Summary of Index Pregnancy:.....

Placenta Examined: YES / NO Abruptio: YES / NO% Infarcts: YES / NO%
 Membranes: Clear / Opaque Cord Abnormalities:

Case Discussion: PNM Team / Multidisciplinary Meeting Date:.....

Placental Histo: Y / N / STA..... Post-Mortem: Y / N / STP.....

Results:

Primary Cause of Death:.....

Final Cause of Death:.....

Secondary Contributing Conditions:.....

