Genetic Basis of Human Disorders of Gonadal Development

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A dissertation submitted in fulfillment of the requirements for the degree of Master of Science in Urology in the Faculty of Health Sciences, at the University of the Cape Town

Cape Town, South Africa, 2017.

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LIST OF ABBREVIATIONS

ACTC1	Actin, alpha, cardiac muscle 1
Akr1b7	Aldo-keto reductase family 1, member B7
AMH	Anti-Mullerian hormone
APC	Adenomatous polyposis coli
AXIN-1	Axis inhibition protein 1
BMP2	Bone morphogenetic protein 2
CBX2	Chromobox 2
CCL7	Chemokine (C-C motif) ligand 7
CDKN1B	Cyclin-dependent kinase inhibitor 1B
COPS2	COP9 signalosome subunit 2
CTNNB1	Catenin (cadherin-associated protein)
CYP3A4	Cytochrome P450, family 3, subfamily A
Cyp11b1	Cytochrome P450, family 11, subfamily b
CYP17A1	Cytochrome P450, family 17, subfamily A
DGD	Disorder of Sexual Development
DHH	Desert Hedgehog
DLG3	Discs, large homolog 3 (Drosophila)
DMRT1	Double sex and mab-3 related transcription...
DNA	Deoxy ribonucleic acid
DSD	Disorder of Sexual Development
DSS	Dosage- sensitive sex reversal
DVL1	Dishevelled segment polarity protein 1
ERK	Extracellular signal-regulated kinases
FGF	Fibroblast growth factor
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog Fhl2
FLNB	Filamin B, beta
FoxL1	Forkhead box L1
FRAT2	Frequently rearranged in advanced T-cell...
FRZB	Frizzled-related protein
GAT1	Glucuronyltransferase 1
GK	Glycerol kinase

GSK3B	Glycogen synthase kinase 3 beta
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3...
IL1RAPL1	Interleukin 1 receptor accessory protein...
LGR4	Leucine-rich repeat containing G protein...
MAGEB1	Melanoma antigen family B, 1
MAP3K4	Mitogen-activated protein kinase kinase kinase 4
Mc2r	Melanocortin 2 receptor
MPG	Male pathway genes
NCOR2	Nuclear receptor corepressor 2
NR0B1	Nuclear receptor subfamily 0, group B
NR2F1	Nuclear receptor subfamily 2, group F
Nr4a1	Nuclear receptor subfamily 4, group A,1
NR5A1	Nuclear receptor subfamily 5, group A,5
NR5A2	Nuclear receptor subfamily 5, group A,2
PABY	Pseudoautosomal boundary of the Y chromosome
PGD2	Prostaglandin D2
POR	P450 (cytochrome) oxidoreductase
Ppap2c	Phosphatidic acid phosphatase type 2C
Rac1	RAS-related C3 botulinum substrate 1
Rhoa	Ras homolog gene family, member A
ROCK2	Rho-associated, coiled-coil containing
RSPO 1	R-spondin-1
RWDD2B	RWD domain containing 2B
SF1	Steroidogenic factor 1
SFRP1	Secreted frizzled-related protein 1
SOX	SRY-box-related (SOX) genes
SPRY4	Sprouty homolog 4
SRY	Sex determining region Y
STAR	Steroidogenic acute regulatory protein
TDF	Testis determining factor
TSPY1	Testis specific protein, Y-linked 1
Wnt1	Wingless-type MMTV integration site family...
WNT4	Wingless-type MMTV integration site family...
WT1	Wilms tumor 1
WWOX	WW domain containing oxidoreductase
Xmmv62	Xenotropic-MCF leukemia virus 62

DECLARATION:

I, Patrick Opoku Manu Maison do hereby declare that the research reported is based on my own independent work and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. Any assistance I obtained is mentioned in the acknowledgement section.

This work has not been reported or published prior to registration for the above mentioned degree

Signed by candidate

Patrick Opoku Manu Maison

2ND October 2017

ABSTRACT

South Africa is unique in the arena of Intersex, in that for unknown reasons we have a very high percentage of **ovotesticular DSD (True Hermaphrodite).** Whereas ovotesticular DSD is the least common cause of hermaphroditism in other parts of the world, it is the most common cause of hermaphroditism in South Africa. There have been several studies in the past to determine the cause of ovotesticular DSD in our population but none of these studies found appropriate answers.

The current state of understanding implicates signaling and signal transduction molecules and transcription factors suggesting that it is likely not all of the genetic factors involved have already been identified.

It was hypothesized that exome sequencing of individuals with DGDs will identify new mutations and genes for these conditions.

Therefore, this study aims to identify additional genes that are associated with ovotesticular DSD. By using a whole-genome sequencing approach we expected to be able to identify rare variants with this condition and determine the prevalence of mutations in these genes in the ovotesticular DSD population.

After obtaining informed consent, blood specimen was obtained from eleven out of fifteen patients who had histological diagnosis of Ovotesticular DSD at the Red Cross War Memorial Hospital over a 10 year period. Blood specimen was also obtained from the biological parents of these children and sent to the Ostrer laboratory for whole genome sequencing and analysis.

At the Ostrer laboratory, high quality DNA was extracted from blood for all of subjects and lymphoblastoid cell lines were created. Following sample preparation using the Illumina library preparation kit, sequencing was accomplished using paired-end sequencing technology on an Illumina HiSeq2000 sequencer. The data from the Illumina sequencers was analyzed first using the Illumina sequencing data analysis pipeline for quality control.

Paired end reads were aligned to the Human Reference Genome (NCBI Build 36) using the BWA software. Each alignment was assigned a mapping quality score by BWA, which is the Phredscaled probability that a read is misaligned. The basic functional annotation of SNPs/Indels is performed by ANNOVAR.

The clinical features of these patients was consistent with those found by other studies on Ovotesticular DSD in South Africa and it also showed the same pattern of variation to the clinical features of Ovotesticular DSD from other parts of the world.

Similar to previous South African studies, this study found no convincing gene mutations as the possible etiology of Ovotesticular DSD in South Africa.

Whiles gene mutations such as duplication of SOX 9 have been found in patients with XX Ovotesticular DSD from outside South Africa, this study could not identify any such mutations. This further adds to the suspicion that the unique features of Ovotesticular DSD in South Africa suggests a different etiology from that of other parts of the world.

In conclusion, the etiology of Ovotesticular DSD in South Africa still remains elusive. It is however possible that a genetic mutation may be found from a more critical analysis of the genome of the patients and their parents.

ACKNOWLEDGEMENT

I would like to acknowledge the following persons for their help and guidance without which this project could not have been possible.

Firstly, my supervisor, Prof. John Lazarus for his support throughout my studies and his guidance in undertaking this project work.

I also wish to thank Prof. Harry Ostrer of the Albert Einstein College of New York for supervising the genetic studies that were performed in this project work.

I am also grateful to my family and friends for their support during the course of this dissertation.

Finally, I want to thank all the patients and their parents who willingly participated in this study. I am very grateful to them.

THANK YOU ALL

CHAPTER ONE

1.1 Rationale for the Study

Disorders of sexual development (DSDs) affect 1 in 10,000 individuals in the population or 10,000 individuals between birth and age 25 in the United States.(1–3) Among these are 46XX testicular DSD (formerly called "XX maleness"), 46XY DSD with partial or complete gonadal dysgenesis, and 46XX ovotesticular DSD (formerly called "true hermaphroditism"). The term "Disorders of sexual development (DSDs)" was selected to differentiate these subjects from others with disorders of sexual development (DSDs) that arise from hormone biosynthetic defects or androgen insensitivity.

These disorders are identified at different times in the life course.(4) They may be recognized at birth due to ambiguous genitalia, or may not be diagnosed until later in life, when puberty is delayed, or there is unanticipated virilization or gynecomastia. While most individuals with DSD s do not suffer from cognitive or general health impairments, the diagnosis of a DSD can have substantial effects on the affected and their families. Depending upon when the DSD is diagnosed, parents may have to make difficult decisions regarding sex assignment and the affected may be assigned a gender of rearing that is discordant for their sex chromosome constitution, possibly resulting in a reassignment. This can lead to gender dysphoria later in life and may result in the affected requiring gender-corrective surgery and/or hormonal therapies. Moreover, the affected may not be the only affected member in the family. Diagnosis of DSD s relies upon multiple sources of information, including clinical findings, gonadal histology, chromosome analysis, and genetic testing of known associated mutations (discussed below). These conditions may be sporadic or inherited in an autosomal dominant, autosomal recessive, X-linked, or Y-linked manner.

South Africa is unique in the arena of Intersex, in that for unknown reasons we have a very high percentage of **ovotesticular** DSD **(True Hermaphrodite).** Whereas ovotesticular DSD is the least common cause of hermaphroditism in other parts of the world, it is the most common cause of hermaphroditism in South Africa. There have been several studies in the past to determine the cause of ovotesticular DSD in our population but none of these studies found appropriate answers.(5,6) These studies looked extensively for environmental, familial and even genetic factors but the cause of ovotesticular DSD in South Africa still remains elusive.

The current state of understanding implicates signaling and signal transduction molecules and transcription factors suggesting that it is likely not all of the genetic factors involved have already been identified.

Therefore, this study aims to identify additional genes that are associated with ovotesticular DSD. By using a whole-genome sequencing approach we will be able to identify rare variants, including structural variants that are associated with this condition and determine the prevalence of mutations in these genes in the ovotesticular DSD population

Genetic Correlates of DGD

Many of the sex-determining genes were identified by studying cases of DSDs. Table 1 provides a summary of genes that have shown association with one or more of the DSD.(1–3),(7–19)

Table 1. 1: Known gonad determining genes based on mutations resulting in abnormal gonadal development

Gene	Co-occurring Features	Chromosomal Region	OMIM Number
ATRX	Alpha-thalassemia/mental retardation/hypotonic facies	Xq21.1	300032
DHH		12q13.12	605423
MAP3K1		5q11.2	600982
NR5A1 (SF1)	Adrenocortical insufficiency	9q33.3	184757
NR0B1 (DAX1)		Xp21.3-p21.2	300473
RSPO1	Palmoplantar hyperkeratosis, squamous cell carcinoma	1p34.3	609595
TSPYL1	Sudden infant death	6q22-q23	604714
WNT4	Dysgenesis of kidneys, adrenals and lung	1p35	603490
WT1	Nephropathy, Wilms tumor	11p13	607102
SOX9	Campomelic/acampomelic dysplasia	17q24.3-q25.1	608160
SRY		Yp11.3	480000

Although the genes listed in Table 1.1 can explain some cases of DSD s, they cannot account for them all. The prevalence of patients with mutations in these genes that could explain their condition is thought to be 25% overall; although other factors, such as epigenetic effects, may explain some of the remaining cases, it is unlikely that all of the genetic factors have already been identified. Therefore, the proposed study aims to identify additional genes that are associated with DSDs. By using a whole-genome sequencing approach we will be able to identify rare variants, including structural variants that are associated with these conditions. Knowledge of these genes can help in understanding the biology of these disorders.

Hypothesis

Exome sequencing of individuals with DSD s will identify new mutations and genes for these conditions. These can be used to improve risk assessment and diagnosis.

1.2 Aims/Objectives

1. To define the genes and causal variants in unique families with multiple cases of DSD s where the genes are unknown employing whole exome sequencing.

2. To determine the prevalence of mutations in these genes in the sporadic DSD population.

CHAPTER TWO

2.0. INTRODUCTION

2.1. Disorder of Sexual Differentiation

Diverse disorders of sexual development exist have been recognized in man since ancient times. The question of what determines whether an organism shall develop into a male or a female has been debated long before science developed. There are many ancient myths of a hermaphrodite origin of man and his subsequent bisection into male and female individuals. According to some rabbinical tradition, Adam and Eve were originally a hermaphrodite being, who was subsequently bisected. Many ancient philosophers, such as Heroditus and Plato, make reference to hermaphrodite beings in their writings.(20)

The term "Hermaphrodite" is derived from the son of Hermes and Aphrodite, called Hermaphroditus, who according to Greek mythology united with the nymph of the Fountain of Salmacis, a female, the two becoming one person and possessing both male and female sexual characteristics.

The idea of males and females originating from hermaphrodite origins agrees well with the embryological evidence. The primitive genital ridge is bipotential, containing components that can form either a testis or an ovary, while two sets of genital ducts, the Mullerian and Mesonephric ducts are the beginnings of the female and male internal genitalia respectively.

It is now know that DSDs arise from errors in sexual development of the early embryo which has the potential to develop as either a male or a female. This results in abnormalities in the gonads and/or other internal organs and/or external genitalia. They were previously referred to as hermaphrodites or intersex however; recent advances in molecular biology and genetic research have provided new insight into the precise mechanisms responsible for sexual differentiation and for specific disorders of sexual development. Hence, the nomenclature has improved in order to promote precise communication among colleagues and respect for the sensitivity of patients by eliminating terms such as pseudohermaphroditism, which have been regarded as pejorative by patients. The term *intersex disorders* have been replaced by *disorders of sex development* (DSDs). It is now known that Sexuality has many aspects to it: chromosomal, gonadal, hormonal, phenotypic, psychological or assumed gender role and sexual orientation. All these components of sexuality are considered in a holistic approach to managing DSDs and not just the phenotypic appearance alone.

The incidence of DSD individuals worldwide is relatively low and the most common DSD anomaly reported in the world is that of 46XX DSD (masculinized female) from Congenital Adrenal Hyperplasia with the Ovotesticular DSD (true hermaphrodite) being the least common variants. However, in his report, van Niekerk's found that Ovotesticular DSD is the most common DSD in South Africa, occurring among the Bantu-speaking people.(20)

True hermaphrodites/ Ovotesticular DSD individuals have both ovarian and testicular tissue. The two tissues may be present in either the same gonad, an ovotestis, or as separate gonads (an ovary or a testis).

2.1.1 Ovotesticular DSD

Salen described the first true hermaphrodite in the literature in 1899 however; Klebs is usually credited with the first scientific consideration of Ovotesticular DSD. (5) By the beginning of the modern cytogenetic era (circa 1958), approximately 200 Ovotesticular DSD had been documented. The diagnosis needs to be confirmed histologically, and gonadal biopsies are therefore a necessary part of the investigations. The pathognomonic histologic feature of true hermaphroditism is the presence of seminiferous tubules and ovarian follicles or oocytes, representing testicular and ovarian tissue, both seen in the same patient. The single commonest gonad seen in such patients is the ovotestis, which combines these 2 sex-opposite tissues in a single gonad. (20)

The most frequent karyotype in these patients is 46, XX or various forms of mosaicism, whereas 46XY is very rarely found. (21,22)

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2.1.2. Classification of Ovotesticular DSDs

Four (4) methods have been used to classify true hermaphrodites. These
Methods are based on the histopathologic findings of the gonads found.

i. Klebs Classification (1876)

This method for classifying true hermaphrodites was found to be impracticable, complex and incomplete. It is, therefore, no longer used.

ii. Hinman Classification (1935)

Most authors use this classification which is in three groups:

a. Group I, bilateral

An individual who has an ovary and a testis, either separate or united as an ovotestis, on both sides.

b. Group II, unilateral

Any person having a testis and an ovary, either separate or united as an ovotestis, on one side, and either a testis or an ovary on the opposite side.

c. Group 1I1, lateral

Any individual with a testis on one side and an ovary on the opposite side.

iii. Jones and Scott Classification (1958)

This method of classification is essentially identical to the Hinman classification.
The only difference is the subdivision of the three varieties into groups.

Table 2.1 Jones and Scott classification

GROUP	GONAD ON ONE SIDE	GONAD ON OPPOSITE SIDE
ALTERNATING/LATERAL Group I	 ovary	 testis
BILATERAL Group IIa Group IIb	 Ovotestis Ovary/testis	 Ovotestis Ovary/testis
UNILATERAL		

Group IIIa	Ovary	Ovotestis
Group IIIb	Ovary	Ovary/testis
Group IIIc	Ovary	Two ovotestis
Group IV	Testis	Ovotestis
Group V	Ovary/testis	No gonad
Group VI	Ovotestis	Not examined

iv. Sasaki and Makino Classification (1960)
Although this method lists the different combinations of gonads, they are not grouped into the three main categories, namely: lateral, bilateral and unilateral. Hence, it is not commonly used.

Table 2.2 Sasaki and Makino classification

GROUP	GONAD ONE SIDE	GONAD OPPOSITE SIDE
Group 1	Ovary	Testis
Group 2	Ovotestis	Ovotestis
Group 3	Ovary	Ovotestis
Group 4	Testis	Ovotestis
Group 5	Ovotestis	No gonad
Group 6	Ovotestis	Not examined

2.1.3. Clinical features of Ovotesticular DSD

2.1.3.1 Presenting symptoms
The external genitalia and internal duct structures of ovotesticular DSD display gradations between male and female. In most patients the external genitalia are ambiguous but masculinized to variable degrees, and 75% are raised as male. Among those raised as male, hypospadias and chordee occur in approximately 80%. Among those patients raised as females, two thirds have clitoromegaly. Virtually all patients have a urogenital sinus, and in most cases a uterus is present. The ovary is found in a normal location, more commonly on the left side. The testis or ovotestis may reside at any point along the path of testicular descent. Testes and ovotestes are more commonly located on the right side(23). Sixty percent of gonads palpable in the inguinal canal or labioscrotal folds are ovotestes, which may be clinically suspected on the basis of a difference in firmness at either end of the gonad, consistent with polar segregation of ovarian and testicular tissue(24).

2.1.3.2. The types of gonads

The three types of gonads that can be found in true hermaphrodites' are an ovary, testis and ovotestis.

i. The ovotestis

The ovotestis is one of the most unique features of Ovotesticular DSD.

The location of the ovotestis, i.e., in the ovarian or scrotal position, seems to be influenced by the proportion of ovarian to testicular tissue in it. The greater the mass of testicular tissue present, the greater the chances that the ovotestis will descend from the ovarian position to the inguinal or scrotal region(20).

Macroscopically, ovotestes can be bilobed or ovoid. If bilobed, testicular and ovarian tissues are joined by a pedicle. If ovoid, the ovarian tissue forms a cap on the testicular parenchyma. The limits between testicular and ovarian parenchyma may be well defined in some cases, while in others the oocytes are spread among seminiferous cords/tubules, or even inside the tubules. The ovarian/testicular proportion of tissue varies widely from one patient to another. The testicular zone neighboring the ovarian component shows significant changes in interstitium and tunica albuginea. The interstitial tissue has ovarian-like stroma, not the characteristic loose connective tissue of a normal testis. The tunica albuginea covering the testicular zone shows poor differentiation with persistence of tubular structures within it or that cross it to reach the mesothelial surface. The mesothelial lining of this zone can be columnar instead of flattened.

ii. The ovary

In the majority of cases the histological features of the ovarian tissue was described as well developed and essentially normal, even when it was part of an ovotestis. Most ovaries found in Ovotesticular DSDs do show signs of ovulation. (25). This is substantiated by reports in the literature of Ovotesticular DSD females giving birth to normal children with normal appearing external genitalia(26–28).

iii. The testis

In contrast, the testicular tissue, from the testis and ovotestis, of Ovotesticular DSD patients is immature with interstitial fibrosis and no, or rare, spermatogonia (29). Spermatogenesis is rarely found and occurs in a separate testis. There is a rare report of a male patient with 46XX/46XYOvotesticular DSD fathering a child. (30).

2.1.3.3 The distribution of gonads

When the gonadal distribution was looked at in 694 Ovotesticular DSDs (data collected from six papers), the unilateral Ovotesticular DSD with an ovary on one side and an ovotestis on the opposite side was present in 33.0% of the cases cited in the literature.

The bilateral (ovotestis on both sides of the body) and lateral (an ovary on one side and a testis on the other) true hermaphrodite each accounted for approximately 25% of cases.

Table 2.3: Gonadal distribution in 694 true hermaphrodites(20–22,25,29,30)(van Niekerk, 1974 [n=24];van Niekerk, 1976 [n=3]; van Niekerk& Retief, 1981b [n=382]; Ramsay *et al*, 1988[n~37]; Hadjiathanasiou *et al*, 1994 [n=22j; Krob *et al*, 1994 [n=226j)

TYPE OF DISTRIBUTION	NUMBER OF PATIENTS	PERCENTAGE (%)
Ovary-Testis	169	24.35
Ovotestis-Ovary	229	33.00
Ovotestis-Ovotestis	176	25.36
Ovotestis-Testis	78	11.24
Ovotestis-?	19	2.74
Others	23	3.31

From the above data (Table 2.3), the cases of Ovotesticular DSD reported in the South African black population, a very different distribution frequency of the gonads would be observed. The 27 patients of van Niekerk(20,31) together with the 37 cases of Ramsay *et al*,(22) showed that the unilateral (ovotestis - ovary/testis) Ovotesticular DSD accounted for 62.50% of the cases. The second most frequent gonadal distribution was that of the bilateral ovotestis (17.19%). The differences observed for the gonadal distribution are statistically significant at the 5% level ($p = 0.0196$). This may imply a different cause of hermaphroditism in the South African black population. Vilain stated that the distribution worldwide is 34% ovotestes/ovary, 29% ovotestes/ovotestes and 25% ovary/testes(32)

Of the 694 true hermaphrodites examined in Table 2.3, the location of the gonad on either the left or right hand side of the body is given for 1363 of the gonads. This information is presented in Table 2.4

A study by Wiesmar and Ramdial in South Africa in 2008 showed the composition of the ovotestis. The ovotestes were divisible on gross appearance into 11% bipolar and 89% mixed types. Histologically, the mixed-type ovotestes have an outer mantle consisting of ovarian tissue, which encapsulated an inner core of 2 distinct types. The first is an admixed ovotestis (constituting 44% of the mixed ovotestes), the central core consisted of gonadal stroma, with scattered foci of separate ovarian and testicular tissue. The second type was the compartmentalized ovotestis (constituting 56% of the mixed ovotestes); here, the outer mantle was thickened in the upper pole and encapsulated a large core of testicular tissue in the lower pole of the gonad. The bipolar ovotestis had a strictly polar distribution of ovarian and testicular tissue, which had an irregularly interdigitating junction between the 2 types of tissue.(33)

Table 2.4: The incidence of gonads occurring on the left or right side in true Hermaphrodites

GONAD	LEFT (%)	RIGHT (%)	TOTAL NUMBER OBSERVED (%)
Ovary	288 (43.11%)	135 (19.42%)	423 (31.03%)
Testis	105 (15.72%)	**159 (22.88%)**	264 (19.37%)
Ovotestis	275(41.17%)	401 (57.70%)	676 (49.60%)

From the data above it is seen that the ovary is more prevalent on the left side, while the testis and ovotestis occur more frequently on the right side of the body. It is also noted that the ovotestis is the most common gonad in the Ovotesticular DSD accounting for 49.60% of the gonads. The second most common gonad is the ovary (31.03%) and the least common is the testis (19.37%).

The asymmetry in the position of ovaries and testes in patients with Ovotesticular DSD is not easily explained in genetic terms, since a testis-determining gene product would be expected to be present in equal quantities on both sides. However, if gonadal differentiation ultimately depends on rates of growth, the question arises whether the asymmetry of gonadal growth in hermaphroditism can be related to an underlying asymmetry of the human body. This possibility can be measured by comparing growth characteristics in right and left gonads of normal human fetuses(34,35). The average differences between left and right gonads (calculated by subtracting values of left gonads from those on the right and expressing the difference as a percentage of the mean for each fetus) were studied. It was found that, on average, right gonads exceed left gonads in the three variables investigated, i.e. weight, protein and DNA contents, and hence in numbers of cells. The mean differences are between 6 and 7 % for testes and about 13 % for ovaries. Since testes exceed ovaries in the same criteria used for assessing growth, the relationship between growth and differentiation in human gonads could be determined. Ovaries are smaller and grow less fast than testes; left gonads are smaller and grow less fast than right gonads. If there were any causal relationship between these phenomena, we would expect left gonads to have a tendency to become ovaries and right gonads to differentiate into testes. In normal development this relationship is of no consequence, since the sex-chromosome constitution will override all other tendencies. However, in Ovotesticular DSD, we witness the breakdown of the sex-chromosomal mechanism, and we also see the bilaterally asymmetrical development of ovaries and testes in accordance with the basic asymmetry of normal gonadal development.

2.1.3.4 Distribution of karyotype

There are three different karyotypes found in patients with ovotesticular DSD; 46XX, 46XY and mosaics with 46XX/XY. The most common is the 46XX ovotesticular DSD as was found by van Niekerk et al upon examination of 195 confirmed ovotesticular DSD(20). 46,XX was the most common karyotype, occurring in 59,5% of the cases; 12.8% of the ovotesticular DSD patients had a 46,XX/46,XY mosaic pattern while the 46,XYkaryotype was found in 12.3% of the patients analyzed. The remaining 15.3% of cases showed different mosaic cell lines(21). 46,XX/46,XY Ovotesticular DSD could result from: (1) fertilization of both an ovum and its polar body; (2) fertilization of each of two ova contained within a single binucleated follicle; (3) fertilization of ova derived from different follicles, followed by fusion; or (4) other related phenomena. The etiology of 46XX/46XY Ovotesticular DSD in humans is usually considered to be chimerism, rather than nondisjunction. After reviewing 283 cases of human ovotesticular DSD published from1980 to 1992, Krob *et al* found the 46,XX karyotype to occur in 70.6% of cases(30).Chromosomal mosaicism (20.2%) was the second most common group and the

46,XYkaryotype accounted for 7% of cases. It is interesting to note that in South Africa, 87 of the 88 patients of black or mixed black origin on whom karyotyping was performed had a 46,XXchromosome complement (21,22). In cases reported from other regions, as summarized by Krob *et al*, 1994, only 52% of the hermaphrodites had a 46,XX karyotype(30). This and the fact that the distribution of gonads differs to that in the rest of the world may be an indication that the aetiology of Ovotesticular DSD in the South African black population is different to that from other parts of the world.

It is important to note from these data that among those with 46XX Ovotesticular DSD, the gonadal distribution was mostly unilateral (37%), i.e., an ovary on one side and an ovotestis on the opposite side of the body. The second most common gonadal distribution 46,XX Ovotesticular DSD was **a** bilateral ovotestis in 28.4% of the cases followed by the lateral gonadal distribution.(25)

2.1.3.5 External genitalia

The development of male type external genitalia is believed to be an effect of dihydrotestosterone. The testicular tissue in the Ovotesticular DSD seems to be capable of differentiating the ipsilateral Wolffian duct, but incapable of fully differentiating male type external genitalia. However, despite this, the sex of rearing of 126 case reports of Ovotesticular DSD in the literature were 73% reared as male and 27% as female.
This may probably be because most Ovotesticular DSD patients have a clitorophallus, measuring between two to eight centimeters long at birth(25).
Absence of either clitoral enlargement or a phallus in Ovotesticular DSD is an exception to the rule. The vast majority of patients have one or the other (20).
Overzier classified various abnormal external genitalia found in DSD cases as Types I, II, III, IV, and V. Type I corresponds to normal female external genitalia whiles in Type II, a common external orifice is present. In Type III, a urogenital sinus is located interior to the clitoris or phallus. Type IV shows an internal urogenital sinus and Type V shows normal male external genitalia. External genitalia of the majority of Ovotesticular DSD reported in the literature were Types III and IV (20).

2.1.4 Diagnosis

The possibility of Ovotesticular DSD should be considered whenever a patient with ambiguous genitalia is being examined. Palpating the gonad which may be present in one of the labioscrotal folds is important(20). In Ovotesticular DSD, the gonad is usually present in the right labioscrotal fold or in the right hemiscrotum. It is also important to palpate carefully for any difference in consistency especially at the polar ends of the gonad. In an ovotestis, the testicular portion feels firm whiles the ovarian portion is quite soft. Cytogenetic studies and hormonal evaluations should be performed. Laparoscopy is done to visualize the internal genitalia and look for gonads which are biopsied for histopathology. Usually, Wolffian structures are found Ipsilateral to a testis and a fallopian tube seen Ipsilateral to an ovary. Either a fallopian tube or ovary is seen Ipsilateral to an ovotestis although usually it is a fallopian tube that is seen. A normal or rudimentary uterus may also be seen at laparoscopy. It is unusual to find both fallopian tube and Wolffian structures Ipsilateral to an ovotestis but this has been reported(36).

The diagnosis of a Ovotesticular DSD is confirmed once ovarian and testicular tissues have been histologically demonstrated on gonadal biopsy.(37)

2.1.5 Sex of rearing

Sex assignment is an issue in these patients, and the considerations are similar to those in DSD in general (38). In the past, there was a tendency for patients with Ovotesticular DSD to be reared as males since most have a phallus at birth. Of 146 Ovotesticular DSD reported in the literature, approximately 75% were reared as males(20). Worldwide, two thirds of all Ovotesticular DSD patients are raised as males (39).
The choice of sex assignment for raising the patients was made according to the age of the patient at diagnosis, the clinical and hormonal data, the degree of virilization of the external genitalia, and the familial and cultural context.
A particular aspect in sex assignment may derive from the fact that the ovarian tissue may be normal enough to produce oocytes. Therefore, female assignment with special attention to the preservation of ovarian tissue may be favored in these cases. To date, there are less than 20 cases of pregnancy reported in patients with ovotesticular DSD raised as females, and the majority have had complications like preterm labor or morbidity related to the delivery process, as a consequence of defects in the female reproductive tract(40).
The decision on sex of rearing in ovotesticular DSD should consider the potential for fertility based on gonadal differentiation and genital development, and assuming the genitalia are, or can be made, consistent with the chosen sex.

2.1.6Management

The management of children with Ovotesticular DSD is a multidisciplinary approach including teams of specialist geneticists, paediatric endocrinologists, paediatric urologists, counselors and the child's parents.

2.1.6.1 Surgical treatment

In order to treat a hermaphrodite effectively, the first prerequisite is to decide on the gender of rearing. Initial gender uncertainty is unsettling and stressful for families. Expediting a thorough assessment and decision is required. Factors that influence gender assignment include the diagnosis, genital appearance, surgical options, need for life- long replacement therapy, the potential for fertility, views of the family, and sometimes the circumstances relating to cultural practices.

When necessary, surgical reconstruction can be done. The surgeon has a responsibility to outline the surgical sequence and subsequent consequences from infancy to adulthood. If the child is to

be raised as a female, removal of all testicular tissue present and labioscrotal reductions can be accomplished in early infancy. Clitoral reduction surgery should only be considered in cases of severe virilization (Prader III, IV, and V) and should be carried out in conjunction, when appropriate, with repair of the common urogenital sinus. As orgasmic function and erectile sensation may be disturbed by clitoral surgery, the surgical procedure should be anatomically based to preserve erectile function and the innervation of the clitoris. Emphasis is on functional outcome rather than a strictly cosmetic appearance. It is generally felt that surgery that is carried out for cosmetic reasons in the first year of life relieves parental distress and improves attachment between the child and the parents(41–44).The systematic evidence for this belief is lacking. Parents now appear to be less inclined to choose surgery for less severe clitoromegaly, which is normally the case in Ovotesticular DSDs being raised as female(45). Vaginal dilatation should not be undertaken before puberty. An absent or inadequate vagina (with rare exceptions) requires a vaginoplasty performed in adolescence when the patient is psychologically motivated and a full partner in the procedure.
If patients are to be raised as males, then various types of hypospadias repair can be done, gonads can be replaced with prostheses, the prepenile scrotum can be reconstructed, and Mullerian structures can be removed with the goal of preserving the vas deferens. There is no evidence that prophylactic removal of asymptomatic discordant structures, such as a utriculus or mullerian remnants, is required although symptoms in future may indicate surgical removal. In the patient assigned a male gender role, care should be taken to identify and remove all ovarian tissue. Partial gonadectomy requires a special comment: patients raised as boys, the ovarian portion needs to be removed before pubertal age to avoid estrogen elevation resulting in gynecomastia and other features of heterosexual pubertal development, and also to prevent complications of cystic follicles that may develop in response to elevated FSH. In patients raised as females, testicular tissue needs to be removed to avoid virilization during puberty. Generally, most ovotestis are excised completely because they may be admixed or because of difficulty with precise cleavage of discordant gonadal tissues. Concerning the risk of tumor development in the testicular portion, it has been reported to be low even if the tissue is dysgenetic, especially in the absence of Y chromosome sequences(46).

2.1.6.2 Sex Steroid Replacement

Most of these patients will require hormone replacement therapy in view of the fact that there is removal of the ovotestis early in infancy. The timing of initiation of puberty may vary but this is an occasion that provides an opportunity to discuss the condition and set a foundation for long term adherence to treatment. Hormonal induction of puberty should attempt to replicate normal pubertal maturation to induce secondary sexual characteristics, a pubertal growth spurt, and optimal bone mineral accumulation, together with psychosocial support for psychosexual maturation(47). Intramuscular depot injections of testosterone esters are commonly used in males; other options include oral testosterone undecanoate, and transdermal preparations are also available(48–50). Females require estrogen supplementation to induce pubertal changes and menses. A progestin is usually added after breakthrough bleeding develops or within one to two years of continuous estrogen. There is no evidence that the addition of cyclic progesterone is beneficial in women without a uterus.

2.1.6.3 Psychosocial management

Psychosocial care provided by mental health staff with expertise in DSD should be an integral part of management in order to promote positive adaptation. This expertise can facilitate team decisions about gender assignment/reassignment, timing of surgery, and sex hormone replacement. Psychosocial screening tools that identify families at risk for maladaptive coping with a child's medical condition are available(51). Once the child is sufficiently developed for a psychological assessment of gender identity, such an evaluation must be included in discussions about gender reassignment. Gender identity development begins before the age of 3 years, but the earliest age at which it can be reliably assessed remains unclear(52). The generalization that the age of 18 months is the upper limit of imposed gender reassignment should be treated with caution and viewed conservatively. Atypical gender role behavior is more common in children with DSD than in the general population but should not be taken as an indicator for gender reassignment. In affected children and adolescents who report significant gender dysphoria, a comprehensive psychological evaluation and an opportunity to explore feelings about gender with a qualified clinician is required over a period of time(53). If the desire to change gender persists, the patient's wish should be supported and may require the input of a specialist skilled in the management of gender change. The process of disclosure concerning facts about karyotype, gonadal status, and prospects for future fertility is a collaborative ongoing action which requires a flexible individual based approach. It should be planned with the parents from the time of diagnosis(54)**.**

2.1.7 Prevalence of 46XX Ovotesticular DSD in South Africa

In southern Africa ambiguous genitalia is more common in blacks than in whites. In a study by Ramsay *et* al 152 black and only 31 white patients were referred to their laboratory, over a four year period, for chromosome analysis because of ambiguous genitalia(22). All thirty eight of the black patients, who were histologically examined, were proven to be Ovotesticular DSD; the remaining 114 had an XX karyotype but were not histologically examined. None of the 31 white patients was confirmed to be an Ovotesticular DSD on histological examination. The majority of white patients were affected with congenital adrenal hyperplasia.
46XX Ovotesticular DSD is thus, unusually prevalent in southern African blacks. This raises concern that there must be some unique factor(s) causing the high incidence of this sexual anomaly in blacks and, it seems likely that the condition has a common etiological and pathogenetic basis in this population. Ovotesticular DSD is the least common intersexual anomaly in the world, however, it is the most common type of intersexuality in the Bantu speaking people of South Africa(20). Thus, there seems to be a special predisposition in them that may account for the high incidence of this disorder.

2.1.7.2. Familial incidence

In general, Ovotesticular DSD occurs sporadically. However, although not common, the occurrence of 46XX Ovotesticular DSD in siblings has been reported in the literature(55).This suggests a possible genetic etiological factor for this condition(20). 46,XX males sometimes co-exist in the same family with Ovotesticular DSD(56,57)

Four families have so far been reported where 46XX Ovotesticular DSD co-exist with 46,XX males suggesting that both disorders are variations of the same genetic defect(58). The presence of male sexual development in genetic females, with transmission through normal male and female parents, indicates that the critical genetic defect may be a new mutation manifesting as an autosomal dominant trait. The different phenotypic manifestations arise from variable expressivity(57) or an X-linked mutation (limited by the presence of the Y chromosome)(59). In these cases a common origin of the sex determining disorder has to be postulated(30).

2.2. The aetiology of 46XX Ovotesticular DSD

The genetic mechanism(s) responsible for 46XX Ovotesticular DSD remains elusive. Ovotesticular DSD is a genetically heterogeneous condition with several possible causes. Three major hypotheses have been proposed to explain the aetiology of Ovotesticular DSD and the phenomenon of testicular differentiation that occurs despite the fact that approximately 60% of these individuals have an XX karyotype and lack the Y chromosome:

i. Mosaicism with a loss of the Y containing cell line, presumably after it had initiated gonadal differentiation into a testis at an early embryonic stage, is the oldest explanation proposed(60–62).

ii. Translocation of chromosomal material encoding the testis determining factor (TDF) from the Y chromosome to an autosome or X chromosome during meiotic division is another possibility. If this were the correct explanation, a high familial incidence would be expected, but few families with XX true hermaphroditism have been described (63).

iii. An autosomal or X-linked dominant mutation that permits testicular determination in the absence of TDF thus allowing XX zygotes to assume a male phenotype(61,62)

There is no direct evidence that a single gene mutation such as this occurs in humans.

The unusually high prevalence of 46XX Ovotesticular DSD in southern African blacks suggests that the condition has a common cause and pathogenesis in this population.

A study by Spurdle et al of 16 46XXOvotesticular DSD patients failed to reveal the presence of the SRY locus and the pseudoautosomal boundary of the Y chromosome (PABY)(64).

Y chromosome sequences are thus very unlikely to be involved in the cause of the disorder in southern African Bantu-speaking blacks. Ramsay *et al* obtained detailed family histories for 11 of their 38 46XX Ovotesticular DSD patients.

They concluded that it is unlikely that mutation of a single gene, either autosomal or sex linked, is responsible for the occurrence of Ovotesticular DSD in the South African black population (22).

2.2.1. Normal fetal sex determination and gonadal differentiation

The most obvious distinction among humans is that of gender. In the history of mankind, both scientists and non-scientists, have always been fascinated and perplexed by the biological processes that determine the sex of an individual(65).
Sexual dimorphism in humans has been the subject of wonder for centuries. In 355 BC, Aristotle postulated that sexual dimorphism arose from differences in the heat of semen at the time of copulation. In his scheme, hot semen generated males, whereas cold semen made females(66). The Greeks believed it was the level of male excitement during intercourse that determined whether the child would be male or female - the more excited the man, the grocer the likelihood of siring a boy(67).
It is now known that sexual differentiation relies on the establishment of chromosomal sex at fertilization, followed by the differentiation of the gonads which ultimately guides the establishment of phenotypic sex. A cascade of complex molecular and morphological events must occur at the appropriate time and in the correct sequence in order for an organism to develop into a 'normal' male or female.
Each event in sexual determination depends on the preceding event, and normally, chromosomal, gonadal, and somatic sex all concur. There are, however, instances where they don't agree, and sexual differentiation is ambiguous, with male and female characteristics combined in a single individual. Analysis of such individuals has permitted identification of some of the genes involved in sex determination (66).

2.2.2. Chromosomal, gonadal and phenotypic sex

Sexual development in the mammalian embryo involves three sequential processes.
The first step, which occurs at fertilization, is to establish chromosomal sex (by the content of the sex chromosomes). Females will normally be XX and males XY, but the number of X chromosomes is irrelevant as the Y behaves as a dominant male determinant. Secondly, the indifferent gonads begin to differentiate into either testes or ovaries (gonadal sex). Ovarian differentiation can be considered the default pathway, which is 'overridden' in males by the testis determining gene, TDF, found on the Y chromosome; and thirdly, the differentiation of internal and external genitalia, that is, the translation of gonadal sex into phenotypic sex(68,69). The pioneering experiments of Alfred Jost in 1953established our understanding about the determining effect of the gonadal ridge development into a testis for the male differentiation of the internal and external genitalia, due to the secretion of testosterone and a second testicular factor, subsequently characterized as the anti-Mullerian hormone (AMH)(70). In their absence, internal and external genitalia follow the female pathway. The degree of virilization of the internal and external genitalia and of the regression of Mullerian ducts depends on the mass of functional testicular tissue present in the narrow window of fetal dimorphic sex differentiation(71). A few years after Jost's work, the determining role of the Y chromosome for testicular development was established(63).

During the indifferent stage, the gonads are bipotential irrespective of their XX or XY karyotype. Gonadal differentiation into a testis or an ovary requires a delicate dosage balance in the timing and levels of expression of several genes(72). In most mammalian embryos, the transient expression of *SRY* , which maps to the Y chromosome, triggers a cascade of gene interactions ultimately leading to the formation of a testis from the indifferent gonadal ridge(73). SRY expression initiates in the middle of the gonad and expands toward the poles(74). The timing and level of SRY expression are critical for proper testis differentiation: delayed or decreased expression results in dysgenetic testicular or ovotesticular differentiation in the mouse (74,75). In most mammals, the SRY-box gene*SOX9* is the earliest upregulated gene in the testis pathway downstream of *SRY* , followed by *CITED4* and other members of the *SOX* family, including *SOX3* , *SOX10* and *SOX13* , and many other genes that are critical for testicular differentiation(72,76,77).
Genes involved in ovarian differentiation of the bipotential gonad increase their expression somewhat later. WNT4 and RSPO1 stabilize β-catenin, encoded by *CTNNB1* , which promotes the expression of ovarian genes, such as *FST* (follistatin) and *FOXL2* . The latter also counteracts SOX9 and other pathways involved in early testis development(72,78,79)

2.2.2. Gonadal differentiation and growth in Ovotesticular DSD

In Ovotesticular DSD, the normal regulation of gonadal differentiation is missing and the bipotentiality of the embryonic gonad persists, with the result that both ovarian and testicular tissues are formed. Gonadal differentiation may revert to a more primitive evolutionary level at which the rate of growth of the gonad determines its sexual direction. When this exceeds a certain threshold a testis may develop despite the absence of the TDF gene(80).
In Ovotesticular DSD there are a number of different combinations of gonads e.g, ovary and testis, bilateral ovotestis, an ovotestis and contralateral ovary/testis. In patients who have two types of gonads present, they are distributed in a bilateral asymmetric fashion. The ovary is more commonly situated on the left side of the body, whereas the testis is more common on the right side of the body. One might, therefore, expect the ovotestes, being part ovary and part testis, to be distributed in a bilaterally symmetrical fashion, but this is not the case. The distribution of the ovotestes resembles that of the testes occurring predominantly on the right hand side.
An ovotestis, therefore, represents a testicular gonad more often than an ovarian one and, like a testis, it is preferentially situated on the right side.
The unequal distribution of ovaries and testes in Ovotesticular DSD cannot be easily explained genetically, as the TDF would be expected to be distributed evenly, and in equal quantities, in the cells of the body. If, however, gonadal differentiation is ultimately based on rates of growth, the question arises whether the asymmetry of gonadal growth in hermaphroditism can be related to an underlying asymmetry of the human body. By comparing growth characteristics in right and left gonads of normal human fetuses this possibility was tested(81). In the three variables investigated, i.e. weight, protein and DNA contents, the right gonads, on average, exceeded the left gonads. By combining the two sets of data, the relationship between growth and differentiation in human gonads can be deduced.
Ovaries are smaller and do not grow as fast as testes; left gonads are smaller and grow

slower than right gonads. If there was any casual relationship between these phenomena, we would expect left gonads to have a tendency to become ovaries and right gonads to differentiate into testes. In normal development this relationship is of no consequence, since the sex chromosome constitution will override all other tendencies.
However, in DSDs, we witness the breakdown of the sex chromosomal mechanism, and we also see the bilaterally asymmetrical development of ovaries and testes in accordance with the basic asymmetry of normal gonadal development(23).
The significance of these results is the observed bilateral asymmetry of gonadal growth in human fetuses provides an explanation for the asymmetrical distribution of ovaries and testes in Ovotesticular DSD.
When testicular tissue differentiates in an *SRY* –negative XX gonad, 2 different mechanisms can be envisaged: the increased expression of pro-testis genes or the insufficient expression of pro-ovarian/anti-testis genes.
An effort to make an accurate diagnosis is important for the provision of proper genetic counseling and long-term management.

2.2.3 Mechanisms Underlying Testis Differentiation in *SRY* -Negative Individuals

2.2.3.1 Increased Expression of Pro-Testis Genes

SOX9

The SOX (SRY-related HMG box) protein family includes a group of transcriptional regulators containing a highly conserved high-mobility-group domain(82). This domain was first identified in *SRY*. *SOX9*, mapping to 17q24.3 in the human, is expressed in several tissues including chondrocytes and testes but also in bile duct, the central nervous system, hair follicles, heart, lung, pancreas, and retina. In the XY mouse embryo, an increase in SOX9 expression is induced by SRY in the gonadal ridge. SOX9 then upregulates FGF9 and PGD2, and a positive feedback loop is established to further upregulate SOX9, which progressively becomes independent of SRY. SOX9 is responsible for Sertoli cell specification, thus initiating testis differentiation(83) and for triggering AMH production(84,85) *SOX9* haploinsufficiency results in dysgenetic 46,XY DSD associated with campomelic dysplasia(6,86). Conversely, ectopic SOX9 expression in undifferentiated gonads of transgenic XX mice results in sex reversal with testis development and male phenotype, demonstrating that high SOX9 expression suffices to trigger testis differentiation in the absence of *Sry* (87).Adult gonads contain seminiferous tubules with Sertoli cells but no spermatogenesis, as expected in a male with2 X chromosomes. Similarly, XX dogs with an interstitial duplication of chromosome 9 which carry the *SOX9* locus develop through the male pathway(88). Testicular and ovotesticular DSD in 46XX *SRY* –negative patients have been described in association with duplications study suggested that the extra dose of SOX9 is sufficient to initiate testis differentiation in the absence of *SRY* ; however, the gonadal histology was not described. A recent case of a boy with normal genitalia has also been reported with a *SOX9* duplication(38). In line with the previous observation and with data in mice identifying relevant *cis* -activating elements within a *SOX9* gonad-specific enhancer (89), duplications or

triplications of potential *SOX9* regulatory sequences have subsequently been identified associated with XX maleness. In fact, a 178-kb duplication 600 kb upstream(90) and a 96-kb triplication 500 kb upstream(91) of the *SOX9* coding sequence were initially identified as the cause of familial 46,XX testicular DSD in *SRY* -negative patients with small testes and azoospermia. Also, a 148-kb tandem duplication of the region –595 to –447 kb upstream of *SOX9* was found in an *SRY* -negative 46XX patient with ovotesticular DSD, who presented with ambiguous genitalia. Two further cases with partially overlapping 17q24.3 duplications 500 kb upstream of *SOX9* presented with dissimilar phenotypes: one was an infertile male with testes, while the other was an ovotesticular DSD with ambiguous genitalia (92). Finally, 3 recent reports have identified 7 further *SRY* -negative patients with 46,XX testicular or ovotesticular DSD, who carried duplications ranging from 68 to 83.8 kb, located 510–600 kb upstream of *SOX9* , which overlapped with previously reported rearrangements and allowed refining the minimal region to a 40.7–41.9-kb element.(93–95)

SOX3

SOX3 is a single exon gene located in Xq27.1, which encodes a protein that is most similar to SRY and that is required for normal brain, pituitary and craniofacial development in mice and humans. The first infant with ambiguous genitalia and scrotal gonads, who carried a de novo mosaic 46,XX,dup(17)(q23.1q24.3) containing the *SOX9* gene, as revealed by FISH, was reported in 1999 (96). The by mutations in humans and mice , its ectopic overexpression in the developing XX gonads provokes testicular development in a transgenic mouse model(97). Elegant experimental studies have shown that, just as SRY, SOX3 can act synergistically with SF1 to upregulate SOX9 expression and trigger Sertoli cell differentiation in the bipotential gonad(98). Concordantly, different rearrangements of the *SOX3* locus have been associated with 46XX testicular DSD, suggesting that overexpression of *SOX3* can cause XX male sex reversal in humans. Duplications encompassing *SOX3* have been reported in 5 *SRY* -negative 46,XX patients with male or ambiguous genitalia and functional evidence of the existence of testicular tissue(92,98,99), although no histological study of the gonads was reported in most of them. In 5 other patients, the condition was associated with rearrangements involving the regulatory region of *SOX3*. A duplication of a common 206-kb region, located 566 kb upstream of the *SOX3* coding sequence, was found in four 46XX patients with testicular DSD(100), while a 773-kb duplication of chromosome 1 integrated 82 kb distal to *SOX3* was described in a patient with testicular differentiation on one side and an ovary on the other side (101). We have recently reported the first case of an *SRY* -negative 46XX patient with ambiguous genitalia associated to a *SOX3* duplication presenting with bilateral ovotestes(102). It should, however, be mentioned that a *SOX3* duplication may not be sufficient to induce testicular differentiation when normal regulatory sequences are lacking in the duplicated DNA fragment (103).

SOX10, a gene closely related to *SOX9*, maps to chromosome22q13.1 in humans and is involved in neural crest and glial development. As for *SOX3* , a functional role for *SOX10* has not been established in normal gonadal development(104), presumably because loss-of-function during gonadal development is likely to be masked by the action of other members of the *SOX* family. However, overexpression of *Sox10* in the gonads of transgenic XX mice results in the development of testes and a male phenotype(105). Interestingly, complete or partial duplications of chromosome22 have been reported in a number of *SRY* -negative XX cases with ovarian

dysgenesis and/or testicular tissue differentiation(105). Relevant to the present review are a patient with masculinized external genitalia,dysgenetic testes and Mullerian remnants with a 47,XX,+22 karyotype(106), a patient with ambiguous genitalia (ovotesticular DSD) associated to an inverted duplication of 22q13.122qter(107), and a patient showing only mild hypospadias and bilaterally palpable testes in whom a duplication of 22q11.222q13 was reported(108).

DMRT1

The *DMRT* gene cluster is located in human chromosome9p24.3 and comprises *DMRT2* , *DMRT3* and*DMRT1* (109). *DMRT* genes have a conserved DNA-binding motif, the DM domain, and play critical roles in gonadal differentiation and gametogenesis. *DMRT1* encodes a male-specific transcription factor and has the most prominent role – amongst *DMRT* genes – in regulating testicular differentiation in all vertebrates. Distal 9p deletions encompassing the *DMRT* cluster are associated with dysgenetic and ovotesticular DSD in 46,XY patients(110). Although no *SRY* -negative 46,XX DSD patients with testicular tissue and *DMRT1* overexpression have as yet been identified, increased expression of *Dmrt1* in the fetal gonads of transgenic XX mice is sufficient to drive testicular differentiation and male sex development (111).

2.2.3.2 Insufficient Expression of Pro-Ovarian Genes

WNT4

WNT4 belongs to the WNT family, a large group of secreted glycoproteins encoded by 19 distinct genes that are expressed in a tissue-specific fashion. *WNT4* maps to 1p36.12, and there is clinical and experimental evidence of its role in sex differentiation. WNT4 cooperates with RSPO1, leading to a reduction in the phosphorylation and degradation of β-catenin. The consequent increase in β-catenin induces WNT-responsive genes and antagonizes SOX9(79,112). WNT4 upregulates DAX1 expression, which antagonizes SF1, and thereby inhibits steroidogenic enzymes. WNT4 overexpression has been associated to sex reversal in a XY patient(3) . Apart from its role in ovarian differentiation, WNT4 is also directly involved in Mullerian duct formation (79) and plays a critical role in the formation of the kidneys, adrenals, pituitary gland, and mammary tissue. The first experimental evidence of the role of WNT4 in sex development derives from the inactivation of the *Wnt4* gene in mice (113). *Wnt4* knockout XX mice are virilised, the Mullerian duct is absent, and the development of the Wolffian ducts resembles that of the male. The gonads are also masculinized, as revealed by the enhanced expression of the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase, required for testosterone synthesis and normally suppressed in the fetal ovary. Heterozygous loss-of-function mutations of *WNT4* have been described in three 46XX patients with mild virilization. The main complaint was primary amenorrhea due to Mullerian defects characteristic of the Mayer-Rokitansky-Kuster-Hauser syndrome and clinical signs of androgen excess(114), but no testicular tissue could be identified(115). Conversely, the inactivation of

both copies of *WNT4* results in a severe clinical condition, known as the SERKAL syndrome, characterized by 46,XX DSD with ambiguous genitalia, renal agenesis, adrenal hypoplasia, and pulmonary and cardiac abnormalities. The presence of testicular DSD and ovotesticular DSD were reported in the same family(116).

RSPO1

RSPO1 is one of the 4 members of the R-spond in family, expressed during fetal development in the central nervous system, the limb buds and the body trunk, and the proximal posterior part of the anterior limb bud(117). The human *RSPO1* gene is located in chromosome 1p34.3. As already mentioned, several lines of evidence have demonstrated that RSPO1 synergizes with WNT4 in XX gonads to stabilize β-catenin, thus acting as an activator of the canonical signaling pathway in ovarian development(79). In mice, the ovarian phenotype of the XX *Rspo1* knockout is strikingly similar to that of *Wnt4* knockout (117). Penetrance of the phenotype is slightly variable, and gonad morphology may range from dysgenetic gonads to ovotestes. Interestingly, β-catenin overexpression is able to rescue the abnormal masculinisation of the gonads of XX *Rspo1* knockout mice, thus confirming that RSPO1 acts through the β-catenin signaling pathway. In humans, the first reported cases with loss-of-function mutations of the *RSPO1* gene were associated with *SRY* -negative 46,XX DSD, palmoplantar hyperkeratosis and squamous cell carcinoma of the skin (19). The presence of 'functional' testes was assumed based on the absence of Mullerian derivatives and the masculinisation of the internal and external genitalia; however, no histological study of the gonads is reported.

A second case with 46XXvirilisation associated with palmoplantar keratoderma, congenital bilateral corneal opacities, onychodystrophy, and hearing impairment was reported later. Laparoscopy showed an apparently normal uterus and left fallopian tube. Histology of the gonad biopsy showed the presence of ovotestis and a seminoma(118). Notably, the normal reproductive phenotype of 46XY individuals suggests that RSPO1 is not required for testis differentiation and function.

FOXL2

FOXL2 is a single exon gene encoding a forkhead/winged helix nuclear protein acting as a transcription factor, and mapping to 3q22.3 in humans. In most vertebrates, FOXL2 is one of the earliest markers of fetal differentiation of the ovary. In mice, *Foxl2* does not seem essential for ovarian development in the XX fetus, but forced expression impairs testis differentiation in the XY fetus(119). FOXL2 continues to be expressed in the postnatal ovary, and ablation of *FOXL2* expression in the XX adult ovary results in its trans differentiation to testis(120) . In 46XX patients, loss-of-function of *FOXL2* is associated with the blepharophimosis/ptosis/epicanthus inversus (BPES)
syndrome with or without ovarian dysgenesis(121), but no development of testicular tissue or signs of virilization have been reported.

2.3. Models for mammalian sex determination

The molecular mechanisms by which SRY triggers testis development are unknown. It is possible for SRY to act as either an activator or repressor of the transcription of target genes. Current knowledge is inconclusive and sometimes contradictory. Genetic models (based on either of the two possibilities) for mammalian sex determination have been proposed.

2.3.1. The SOX model

Since haploinsufficiency of SOX9 promotes ovarian rather than testicular differentiation, it has been proposed that inhibition of SOX9 in the genital ridge must therefore suppress testis formation and lead to a female phenotype. Because the presence of SRY promotes testis formation, a highly conserved gene, SOX3, has been suggested to provide the link between SRY and SOX9(122).

Sox9 is expressed in the gonads of both *XX* and XY*mice* at 10.5dpc. At the onset of Sry expression, Sox9 is upregulated in XY embryos, and remains level or decreases in XX embryos. Sox3 is expressed at a low level in the gonad up to 11.5dpc, as is Sry. These patterns are compatible with competitive inhibition of Sox3 protein by Sry protein in binding to and regulating transcription of the Sox9 gene. In XX embryos, Sox S protein binds to and inhibits Sox Q transcription. In XY embryos, Sry protein would compete with Sox S binding and relieve this inhibition, permitting transcription of Sox9 and initiating testis differentiation (123).

One of the more recent models proposed by (124) suggest that an autosomal testis repressor exists (the *Z* gene) that is active in females but that is itself repressed by SRY in males during sex determination.

2.3.2. The Z gene model

The Z gene model implies that a gene(s) other than SRY directly controls target genes in the male pathway, a view contradicted by the more usually accepted hypothesis that SRY directly activates a testis-determining cascade. This autosomal locus, Z, that confers male phenotypes when both copies of the recessive allele are present, even when SRY is absent, was the simplest way to interpret three pedigrees where the parents exhibited normal phenotypes, but in each family, two of the children were XX sex reversed males or 46,XX Ovotesticular DSDs(124).

To explain the genetic observations - that SRY is not needed for male sex determination in the absence of Z - SRY has been proposed to antagonize or repress Z. In normal XX females, Z negatively regulates the expression of male specific genes.

In normal XY males, SRY represses synthesis of Z or inhibits Z activity, thereby allowing expression of male specific genes, leading to testis development and a male phenotype. In XX patients who lack Z due to a mutation, inhibition of male specific genes does not occur, and, therefore, these XX individuals exhibit a male phenotype(124). XY individuals exhibiting a female phenotype can be explained in terms of a dominant mutation in Z, Z', which is insensitive

to SRY action. McEireavey *et al* proposed the existence of several 'leaky' Z mutants that conserve some residual activity, to explain the variability in sex phenotypes among XX sex reversed individuals.(124)

2.3.3. The DSS-gene model

Jimenez *et al* proposed that a single active copy of DSS (dosage- sensitive sex reversal) may function to silence male pathway genes (MPGs) in normal XX females, permitting the induction of the ovarian pathway (123). This DSS gene model proposes that SRY inhibits the action of the copy of DSS present in XY individuals, thus permitting testis, differentiation. This implies that the Z and DSS genes may be identical, and that Z is not autosomal as originally proposed, but X-linked as is DSS. A single copy of SRY, however, may be unable to counteract the effect of two active copies of DSS, so that male to female sex reversal would occur, as observed in humans with duplications of Xp21. SF1 and WT1 induce the formation of the bipotential gonads in the embryos of either sex chromosome constitution. In XY individuals, SRYsuppresses the repression by DAX1 of SF1. As a result, SF1 is able to transactivate MPGs, including SOX9 and AMH (anti Mullerian Hormone). The resultant differentiation of the testes leads to inhibition of the female pathway genes (FPGs) and hormonal induction of the male phenotype. In XX individuals, the active copy of DAX1 represses SF1 and, therefore, this gene cannot activate the MPGs. Later in development, activation of FPGs leads to the development of ovaries and the consequent hormonal induction of the female phenotype. Functional oocytes may be necessary to inhibit MPGs, thus precluding transdifferentiation of the ovarian tissue into testicular tissue as a consequence of late activation of MPGs(125).

Evidence for the involvement of autosomal and X-linked genes in several sex reversed syndromes in humans and other mammals suggests that the pathway is more complex than the models described above and involves genes that have functions other than sex determination. This complexity and pleiomorphism should not be surprising by analogy with the sex determination pathways of *Drosophila melanogaster and Caenorhabditis elegans.* Indeed, it would be surprising if mammalian sex determination were really as simple as it was naively supposed a decade ago(123).

2.3. 4 A proposed cascade for sex determination

A possible cascade of events, initiated by SRY, which results in testis formation has been proposed. Included in this cascade is SOX9, a hypothetical negative regulator of male development. Z and SF1 which binds to the MIS promoter and thus activates MIS.(124)The proposed sequence of events in a normal XY male, are as follows the expression of SRY triggers the cascade by activating a downstream gene, SOX9.SOX9, in turn, represses Z which (directly or indirectly) inhibits SF1 expression. In the absence of Z, SF1 expression is induced and hence MIS is activated. This results in regression of the Mullerian ducts and consequent formation of the testis.

2.4 A hypothesis on the molecular aetiology of Ovotesticular DSD

2.4.1. The X chromosome inactivation theory

In 1961, Mary Lyon postulated that dosage compensation for X-linked genes in mammals is accomplished by inactivating one of the two X chromosomes in females.
X-inactivation occurs early in embryogenesis, at the 64 to 100 cell stage. It is thought to be a stochastic event; that is, each cell is equally likely to inactivate either the maternal or paternal chromosome. Neighboring cells do not influence the chromosome 'choice'. Therefore, in normal females, approximately 50% of the cells transcribe genes on the maternal X chromosome (126). Some loci on the inactivate X chromosome are, however, transcribed. This late-replicating chromosome is relatively condensed during interphase and is visible as a Barr body at the nuclear membrane.
The process of X-inactivation involves a regulatory gene, Xist (X-inactivation specific transcript). It is now known to represent the master switch locus regulating X inactivation. Xist maps to the X-inactivation centre (Xie) and is transcribed exclusively from the inactive X chromosome in female somatic cells and at the time of X-inactivation during spermatogenesis in the male (127). The RNA remains in the nucleus and coats the chromosome. X chromosome inactivation is a complex developmental process that is likely to involve a variety of molecular mechanisms. In addition to the mechanisms that initiate inactivation, other mechanisms act to maintain it with high fidelity during cell division.
Although several models have been proposed to explain the mechanism of X inactivation at a molecular level, experimental evidence in support of these theories is lacking(128).
An excellent candidate, however, for the mechanism that silences genes on the inactive X chromosome is DNA methylation. CpG islands on the active X are unmethylated whereas those on the inactive X are extensively methylated (128).

Thus, it's been proposed that testis formation can occur in an individual with a 46XX karyotype if a gene necessary for sex determination is mutated and is present and on the active X chromosome. The normal copy of the gene is found on the inactive methylated X chromosome and is therefore not expressed. This hypothesis is thus based on whether X chromosome inactivation in the testicular and ovarian tissue of the gonads is random or non-random in XX Ovotesticular DSDs. Non-random inactivation of either the maternal or paternal allele in either the testis or ovary would suggest that there is a gene(s) on the X chromosome which plays a role in testicular development in XX Ovotesticular DSDs. The basis of the X inactivation assay lies in the fact that one of the two X chromosomes in every female cell becomes inactivated in early fetal development. This is a random process that results in a mosaic pattern of X inactivation. The active and inactive X chromosomes may be distinguished from one another, in this case, on the basis of methylation. This X chromosome inactivation hypothesis can be assessed by examination of the methylation status of the CpG region near the polymorphic androgen receptor element (ARE) gene on the X chromosome.
The method relies on methylation-sensitive restriction enzyme digestion and PCR. Methylated DNA sequences i.e., inactive X chromosomes, are selectively amplified

following digestion with *HpaW.* This assay is able to distinguish between the paternal and maternal alleles and to identify their methylation status. Thus it is possible to determine whether there is random or non-random inactivation of the X chromosomes in Ovotesticular DSD

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Site
The screening and of all study participants and drawing of their blood specimen were done at the Red Cross War Memorial Hospital in Cape –Town, South Africa. The blood specimen were then packaged appropriately and couriered by FedEx on the same day for whole genome sequencing by our partners at the Albert Einstein College of Medicine in New York, USA.

3.2 Study Participants
Participants enrolled into the study were patients who had histologically proven ovotesticular DSD and their biological parents. Blood specimens were also drawn from other healthy black individuals of the Bantu- speaking ethnicity to be used for controls.

3.2. 1 Inclusion Criteria
1. Patients with histologically proven ovotesticular DSD and their biological parents as study cases
2. Healthy individuals from the Bantu speaking ethnic group who consented for their bloods to be taken as control

3.2.2 Exclusion Criteria
Patients diagnosed with DSD but without histologic confirmation of ovotestis or the presence of an ovary and testis.

3.3 Study Design
This was a prospective analytical study of patients with diagnosed with ovotesticular DSD at the Red Cross War Memorial Hospital and their biological parents. Hospital records were reviewed to select patients who had been diagnosed with ovotesticular DSD over the past 10 years (January 2007- December 2016). These were patients who had undergone gonadal biopsy as part of the work up for disorder of sexual differentiation and had histological evidence of seminiferous tubules and ovarian follicles present in both or either of their gonads. Their records were reviewed for data such as features of internal and external genitalia, hormonal assay results, histology of gonads and sex of rearing etc. The parents of these patients were contacted on phone. Those who could be reached were informed of the study and if they agreed to partake in the study, they were scheduled to report to the Red Cross Hospital with the patient for informed consent and blood specimen to be taken. In the event where only one parent was available with

the patient, blood specimen were drawn from only the two of them or if both biological parents were not available, blood was drawn from the child alone.
Blood specimens were drawn from each study participant into specimen tubes, labeled, tubes taped to prevent leakages and packaged for courier by FedEx to New York for genome sequencing.
At the genetic pathology laboratory of the Albert Einstein College of Medicine, cell lines were created for each study participant and DNA isolated and quantified by Nanodrop technique. Then exome sequencing and functional studies were carried out.
.

3.4Sample/ Data Management:
All samples were assigned a unique ID, consisting of a study identifier, family identifier, and sample identifier. This ID is used for all processes. A master file is kept by the research genetic counselor to match the study ID to the subject's identifying information. This file is password-protected and stored on a secure server at Albert Einstein College of Medicine.

All other study data were stored in a secure database on the server at Albert Einstein College of Medicine.

High quality DNA was extracted from blood for all of subjects. Lymphoblastoid cell lines were created for the selected cases. To create lymphoblastoid cell lines, white blood cells were separated from 10 ml of whole blood from each participant, using a ficoll-hypaque gradient and then transformed in culture by infection with Epstein-Barr virus to create a renewable source of DNA, RNA and actively dividing cells.

3.4.1 Massively parallel sequencing.
Following sample preparation using the Illumina library preparation kit, sequencing was accomplished using paired-end sequencing technology on an Illumina HiSeq2000 sequencer. Each sample was sequenced to between 30 and 35-fold coverage. Experience has suggested that this is an appropriate level of coverage for whole exome sequencing to capture most of the SNVs and small indels with high confidence. The data from the Illumina sequencers was analyzed first using the Illumina sequencing data analysis pipeline for quality control. For low sequencing error rates, this has been deemed to be sufficient for identifying low frequency variants.
The following criteria are used to call SNPs and indels:
1. Minimum root-mean-square mapping quality for SNPs = 25
2. Minimum root-mean-square mapping quality for gaps =10
3. Minimum read depth for SNV and indels = 3
4. Maximum read depth for SNVs and indels = 256
5. No more than 2 SNVs in a 10 base pair window
6. No more than 1 gap in a 30 base pair window
7. SNVs within 10 base pairs of an indel of quality 25 or higher will be filtered out.

Paired end reads were aligned to the Human Reference Genome (NCBI Build 36) using the BWA software. Each alignment will be assigned a mapping quality score by BWA, which is the

Phredscaled probability that a read is misaligned. To call SNPs or indels, the Unified Genotyper of GATK was applied. Due to the fairly aggressive manner of the Unified Genotyper in making either SNP or Indel calls, the raw call sets were filtered to reduce false positive results based on Call Quality, Depth and Strand Bias etc. The basic functional annotation of SNPs/Indels was performed by ANNOVAR. The program determines whether the variants are located in exons, introns, ncRNA or intergenic regions and lists the related genes. If the variants are within exonic regions, the functional consequences of the variant are appended, including nonsynonymous single nucleotide variant, synonymous single nucleotide variant, frameshift insertion, frameshift deletion, nonframeshift insertion, nonframeshift deletion, frameshift block substitution, nonframeshift block substitution, stopgain and stoploss. For nonsynonymous DNA variants, the effect of the amino acid change will be predicted using BLOSUM62, SIFT, and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/).

For a known SNP from the 1000 Genomes Project32 or from dbSNP, the alternative allele frequency or heterozygosity, respectively, will be added to our annotation. At this stage we will have a series of annotations, each of which provides a measure of the likelihood that sequence changes are of functional significance. By scanning for their presence in the Human Gene Mutation Database at the University of Cardiff, Wales (http://www.hgmd.cf.ac.uk) or at the newly created MutaDatabase (http://www.mutadatabase.org), it was determined whether these variants had been described previously. From studying 3 affecteds within a family, we expect that as few as 1-2 filtered variants and as many as 17 filtered variants may be shared among the affecteds.

For *de novo* mutations, after correcting for sequencing errors and excluding SNPs or mutations of known frequency and significance, we may expected to have as few as 1-2 or as many as 5-10 filtered variants. We anticipated that the causal mutations will be very rare in the population and not necessarily the same from case to case. Most may cluster in certain genes or pathways. For this reason, we looked for evidence of an excess of rare functional mutations in known developmental pathways, including those that have been implicated in prior linkage and genomic sequencing studies.

To confirm the identity of the top 20 variants in each family and to perform linkage analysis, we performed TaqManq PCR genotyping assays on the affects and all other available members of the family. Thus, it was possible to determine which variants showed putative linkage to the phenotype (LOD score >1.0). In addition, for the sporadic cases, it was possible to demonstrate that the mutation is indeed *denovo*. This served as a basis for looking for functional effects.

3.4.2 Functional effects of mutations.

The expertise in the Ostrer laboratory and in the core facilities at Albert Einstein College of Medicine was tapped to use a toolkit of assays for rapidly screening missense, splicing and regulatory variants. These methods were also applied efficiently to studying small and large indel mutations. The first step was to assess the variant and determine whether it was likely to affect

RNA or protein abundance, splicing, targeting or interaction with co-factors. The choice of assay was based on the variant observed. Some of these analyses were performed for mutations known to cause DGDs, thus providing positive controls for these experiments. These assays were applied to lymphoblastoid cell lines that were derived from patients. Mutant and wild-type cell lines from patients were used to assess the effects of mutations on transcripts and proteins, if the repertoire of genes is expressed in this cell type.
qPCR experiments were designed and performed to test effects of variants on RNA abundance using ABI Taqman expression probes. The experiments were designed not only to test the abundance of the various human isoforms of these transcripts, but also to test for aberrant splicing. If alternative splicing was suspected, then the qPCR products were cloned and sequenced to identify the effects of the aberrant splicing.

Proteins were quantified and analyzed for protein-protein interactions by Western blot analysis using infrared fluorescence reporting secondary antibodies that generates signals for different epitopes. For example, these imaging systems quantify the phosphorylated and the non-phosphorylated forms of proteins and the internal loading standard, such as histone, α-tubulin or β-actin. Multiple proteins can be analyzed simultaneously by the use of primary antibodies from different species, which increase the accuracy of quantification and comparison. Moreover, we have shown that the efficiency of these methods can be enhanced by performing FCM-Western analysis. Processing with secondary antibodies occurred according to manufacturer's protocol and selection of primary antibodies. Sufficient biological replicates were performed to achieve statistical analysis and graphical representation. The intensities for each protein was normalized to the suitable loading controls and results presented as box plots of mean and standard deviation, and then compared between groups by Student's t-test. Significant results were confirmed by conventional Western blot analysis. As with the RNA analysis, statistically significant increases or decreases in variant versus wild-type was taken as indication of a positive result. Notably, FCM-Western analysis was performed in a 96 or 384-well format, allowing for testing of multiple replicates and multiple different epitopes simultaneously, thus markedly enhancing the throughput.

Binding partners for co-immunoprecipitation experiments were chosen from published reports and pathway analysis and selected on the basis of interacting with domains in which a variant amino acid residue had been identified. Suitably reactive antibodies were selected from published reports and the Albert Einstein College of Medicine Analytical Imaging Core. An antibody for the protein of interest was incubated with a cell extract in a non-denaturing buffer in the presence of protease and phosphatase inhibitors, so that the bait antibody will bind the protein along with its complex in solution.

The lysate was pre-cleared by binding to a non-specific antibody and the antibody/antigen complex pulled out of the sample using protein A or G-coupled magnetic Dynabeads, which physically isolates the protein of interest from the rest of the sample. Protein G-coupled Dynabeads was used for monoclonal antibodies and protein A-coupled beads for polyclonal antibodies. The sample was then bound to antibody-conjugated beads for Western analysis, using the methods described above.

Population prevalence. To determine the prevalence of these mutations in the ethnic population for each of these families, 200 samples previously collected under anonymized specimen study protocols were genotyped from the corresponding group – Hispanic/Latino, Dominican, Ashkenazi Jewish, African American and European. These genotyping assays were created with suitable positive and negative controls. Should they have significance for the health of the population involved, approval will be sought from the New York State Department of Health for clinical use of these validated assays

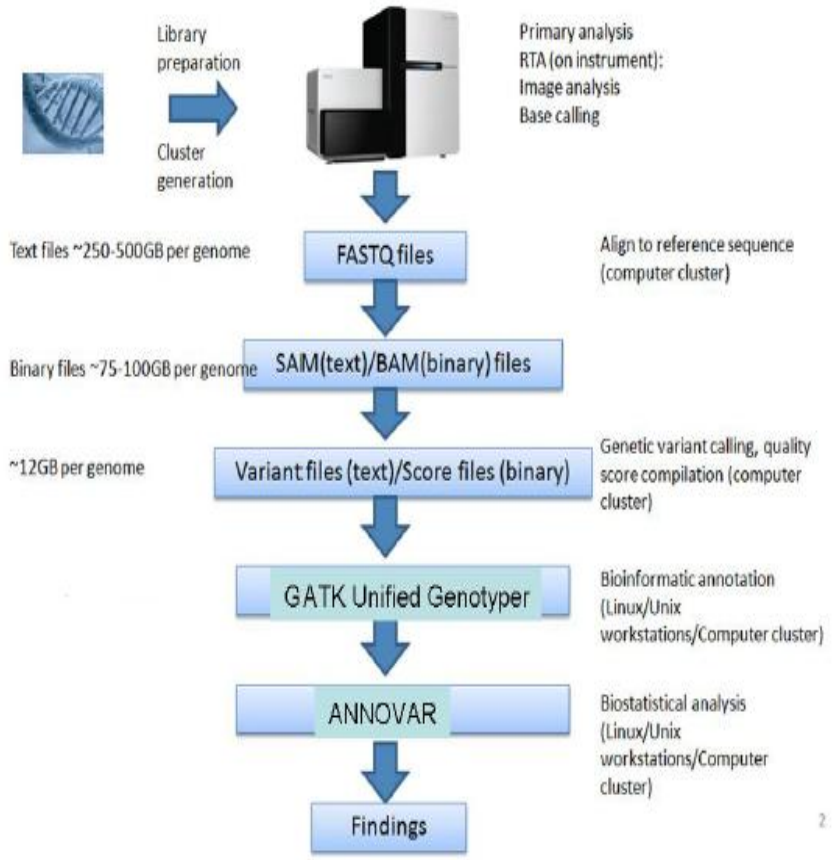


Figure 2: Whole exome sequencing strategy

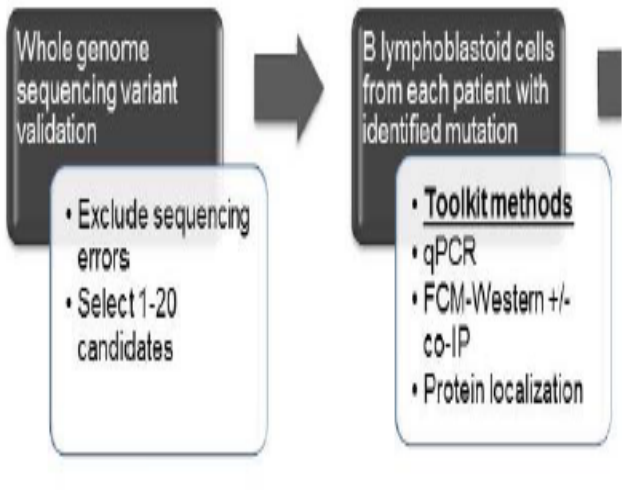


Figure 3: Strategy for assessing the phenotypic effects of mutations

3.5 Data Analysis

Gene analysis was done for mutations in the genome of patients (probands) and their mothers.

1. Dominant gain in function mutation:

Prediction: One variant found in all probands and in no mothers. The genes looked for were the testis promoting genes.

Gene list: SRY, RAC1, MAP3K4, AXIN1, SOX3, SOX8, SOX9, FGF9, FGFR2, SRY, GADD45Y

Examples of predictions:

(varX is pathogenic).

Probands	Mothers
wt/varX	wt/wt
	wt
	wt/varY
	varY
	varY/varY
varX/varY	wt/wt
	wt
	wt/varY
	varY
	varY/varY

2. Haploinsufficiency (Dominant)

Prediction: One variant found in all probands and in no mothers. The genes looked for were the genes which cause down regulation of testis development.

Gene List: RHOA, ROK, ERK1, ERK2, FRAT1, P38

Examples of predictions:

(varX is pathogenic)

Probands	Mothers
wt/varX	wt/wt
	wt
	wt/varY
	varY
	varY/varY
varX/varY	wt/wt
	wt
	wt/varY
	varY
	varY/varY

1. Dominant (Other)

Prediction: One variant found in all probands and in no mothers. All genes related to DSD were looked at for mutations

Examples of predictions:
(varX is pathogenic)
.

Probands	Mothers
wt/varX	wt/wt
	wt
	wt/varY
	varY
	varY/varY
varX/varY	wt/wt
	wt
	wt/varY
	varY
	varY/varY

Triage filters (Cartagania)

- **Population Frequency**
  - ≤5% frequency in any of the following:
    - ESP6500
    - ExAC
    - 1000 Genomes Phase 1
    - 1000 Genomes Phase 3
    - dbSNP
    - **Coding Effect**
  - Excluding Synonymous mutations
- **Genes/Regions**
  - Gene List (Total DSD)
  - **Cohort**
  - Keep variants that are present in all probands as:
    - Heterozygous wt / var x
    - Heterozygous var x / var y
    - Hemizygous (?)
    - Homozygous var x / var x (UPD?)
  - Exclude variants that are present in all mothers as:
    - Heterozygous wt / var
    - Heterozygous var x / var y
    - Hemizygous (?)
    - Homozygous var x/ var x (UPD?)

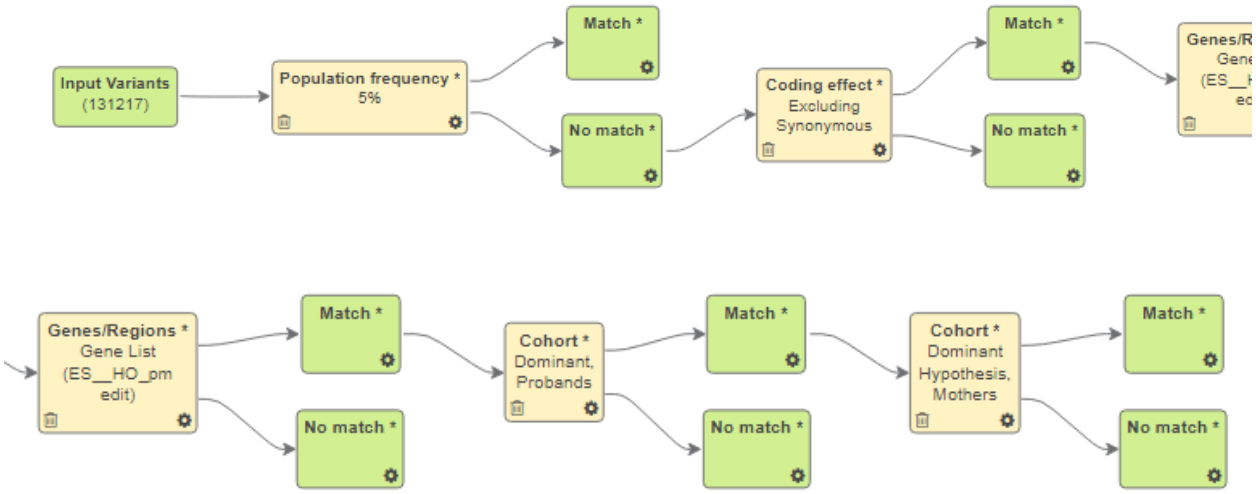


Fig 3.1 Diagram of analysis for mutant dominant gene

1. Recessive

Prediction: Variant found on both alleles in probands and only on one allele in mothers.

Examples of predictions:
(varX is pathogenic)

Probands	Mothers
varx/varx	wt/varx
	wt/wt
	varX/varY
	varY/varY
	wt/varY
	varY
	wt

Triage filters (Cartagenia)

- **Population Frequency**
  - ≤5% frequency in any of the following:

        - ESP6500
        - ExAC
        - 1000 Genomes Phase 1
        - 1000 Genomes Phase 3
        - dbSNP
        - **Coding Effect**
    - Excluding Synonymous mutations
- **Genes/Regions**
    - Gene List (Total DSD)
    - **Cohort**
    - Keep variants that are present in all probands as:
        - Homozygous var x/ var x
        - Hemizygous (?)
    - Exclude variants that are present in all mothers as:
        - Homozygous var x/ var x
        - Hemizygous (?)

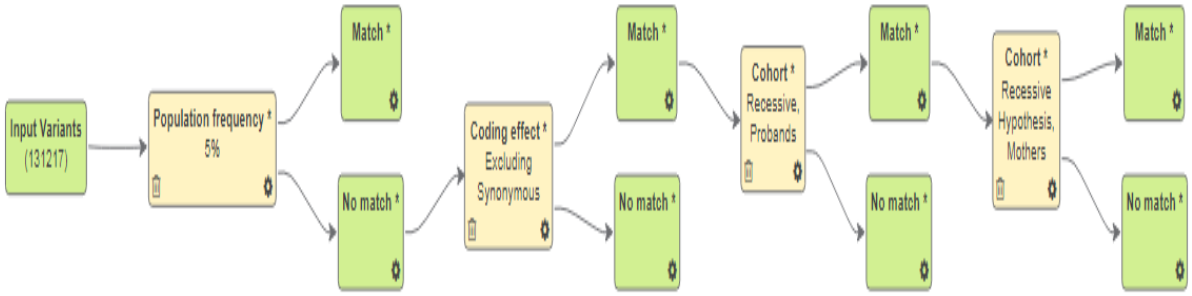


Fig 3.2 Diagram of analysis for mutant recessive gene

3.6 Ethical and legal considerations

Ethical clearance for the study was obtained from the Human Research Ethics Committee (HREC) of the University of Cape Town (UCT). Permission was initially obtained from the Departmental Research Committee of the Red Cross Hospital. The Departmental Research Committee of the Red Cross Hospital is the office that coordinates, registers and supervises all research works that take place in the hospital. This office was first notified of the study with an application letter and the research proposal. Registration of the research and permission for it to be conducted in the hospital was granted after the research proposal was approved. Then the HREC of the UCT and Ethics was contacted for ethical clearance which it granted after studying the research protocol and ensuring that the highest ethical standards were to be maintained in conducting the study.

A written informed consent (Appendix I) was obtained from all study participants after a general description of the study and the essence of their participation was verbally explained to them in a language they understood. Informed consent for patients who were less than 18years was obtained from their parents or guardians although the patient himself was also informed for his permission if he/she was old enough to understand. Patients were assured that participation was voluntary and that refusal to participate in the study was not going to affect management. Once the study genetic results were ready, a genetic counselor will link results back to the family and individual participants for appropriate genetic counseling. Patients were also assured of confidentiality in the handling of the data to be obtained from them. Information obtained from the study participants and the results of gene sequencing was used for no other purpose other than the study and only the researchers have access to it. It shall be retained for at least 5years before being destroyed.

CHAPTER FOUR

4.0 RESULTS

The retrospective review of 30 patients' files, revealed 15 patients with ovotesticular DSD. Seven (7) of these patients and their biological parents consented and had blood specimen taken for whole gene analysis.

Clinical Findings

Thirteen of these 15 patients have a 46XX karyotype while the karyotype of 2 patients was unknown. The sex of rearing for the 15 ovotesticular DSD patients is 40% male and 60% female as shown in figure 1. As shown in figure 2, 53% of patients presented with hypospadias while 7% did not have hypospadias (the presence or absence of hypospadias was not recorded for 40% of patients). Furthermore, 33.3% of the patients had palpable gonads (5 out of 15), with 40% (2 out of 5) being on the right and 60% (3 out of 5) being on the left. No patient had bilateral palpable gonads. 53.3% of patients did not have a palpable gonad.

Histologic Findings

All 15 patients had undergone a gonadal biopsy at Red Cross War Memorial Children's hospital to confirm the diagnosis. This biopsy found ovotestes to be the most common gonad, accounting for 63.3% of gonads. Ovaries accounted for 26.7% of gonads while testes accounted for 10% of gonads.

Gonadal Laterality and Distribution

Furthermore, 14 out of 19 ovotestes (73.7%) were found on the right side of the patient's body while 7 out of 8 ovaries were found on the left side of the patient's body. Four patients (26.7%) presented with bilateral ovotestes. Bilateral ovotestis indicates that ovotestes were found on the left and right ie. 8 ovotestes were present and 4 sets of bilateral ovotestes were seen. Eight patients (53.3%) had an ovotestis and ovary with 7 of those patients presenting with the ovary on the left (46.7%). Three patients (20%) had an ovotestis and testis with all 3 patients presenting with the testis on the right. No patient presented with an ovary on one side and a testis on the other.

Laparoscopic Findings

At laparoscopy, it was found that 10 out of 15 (66.7%) patients had a uterus, with 5 out of 10 (50%) presenting with an abnormal uterus and the remaining 50% with a normal uterus. It was also found that 7 out of 15 patients had one or both fallopian tubes. Five out of 7 (71.4%) patients presented with normal fallopian tubes while the remaining 2 patients (28.6%) had

abnormal fallopian tubes. There was no comment on the presence or absence of fallopian tubes in the remaining 8 patients.

Figure 4.1. Sex of Rearing

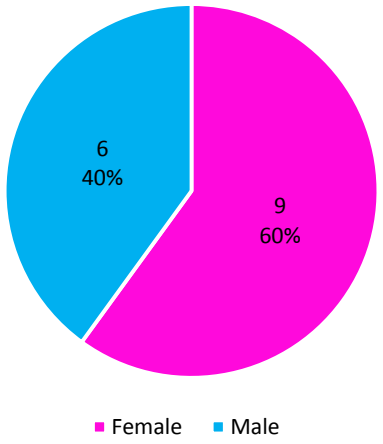


Figure 4.2.
Hypospadias

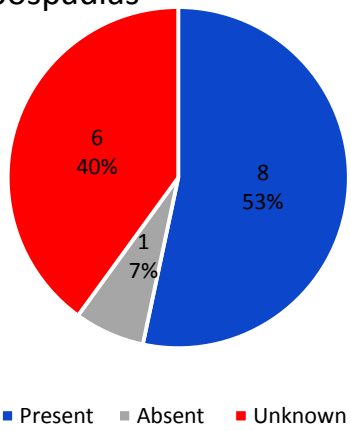


Figure 4.3. Palpable Gonad

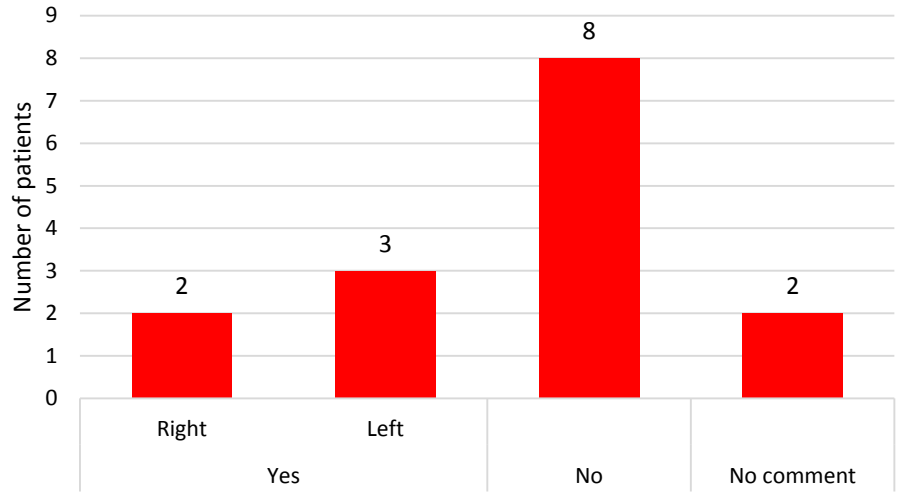
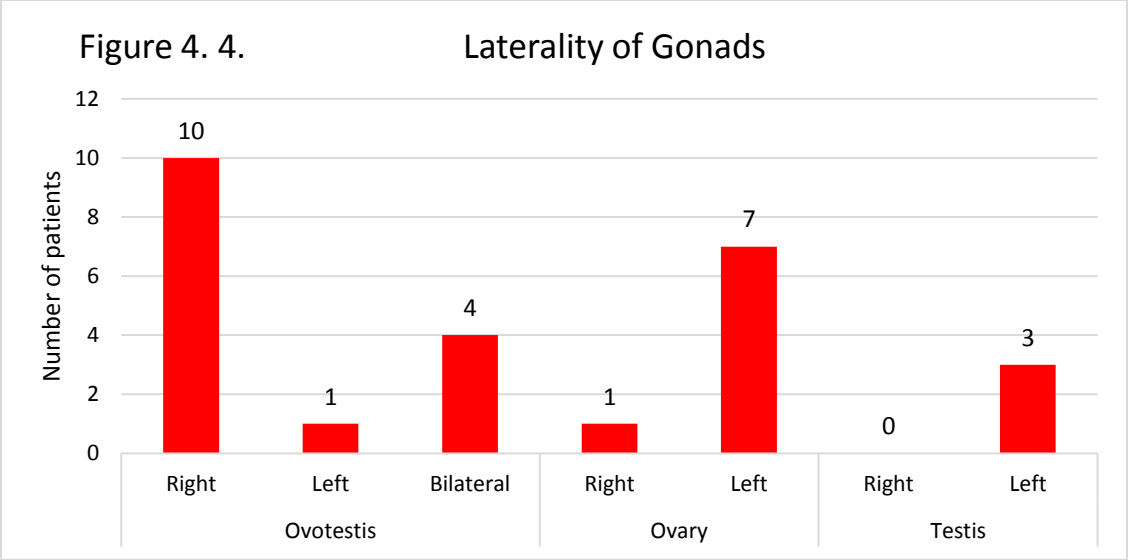


Table 4.1. **Table to show the type of gonad found.**

Gonad	**Number of gonads**	**(%)**
Ovotestis	19	63.3
Ovary	8	26.7
Testis	3	10
Total	30	100



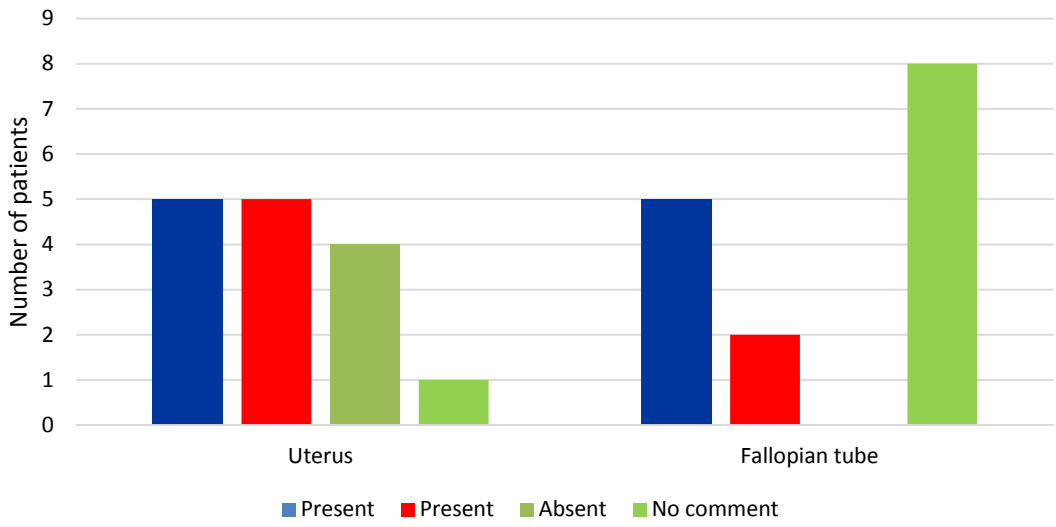
. Table 4.2 Laterality of gonads in percentages.

Gonad	Number on right (%)	Number on left (%)
Ovotestis	14 (46.7%)	5 (16.7%)
Ovary	1 (3.3%)	7 (23.3%)
Testis	0	3 (10%)
Total	15 (50%)	15 (50%)

Table 4.3 Distribution of gonads.

Left gonad	Right gonad	Total number of patients	Percentage of patients (%)
Ovotestis	Ovotestis	4	26.7
Ovotestis	Ovary	1	6.7
Ovary	Ovotestis	7	46.7
Ovotestis	Testis	3	20
Testis	Ovotestis	0	0
Testis	Ovary	0	0
Ovary	Testis	0	0
Total		15	100

Figure 4.5 Laparoscopic presentation of uterus and fallopian tubes



B. Summary of results of whole genome sequencing.

Seven (7) patients with histological confirmation of Ovotesticular DSD and their biological parents consented to have blood specimen taken for whole-gene analysis.

Gene analysis was done for mutations in the genome of patients (probands) and their mothers.

2. Dominant gain in function mutation:

Prediction: One variant found in all probands and in no mothers. The genes looked for were the testis promoting genes.

Gene list: SRY, RAC1, MAP3K4, AXIN1, SOX3, SOX8, SOX9, FGF9, FGFR2, SRY,GAD D45Y

The results did not find a mutation in any of these genes present in all probands.

3. Haploinsufficiency (Dominant)

Prediction: One variant found in all probands and in no mothers. The genes looked for were the genes which cause down regulation of testis development.

Gene List: RHOA, ROK, ERK1, ERK2, FRAT1, P38

The results did not find a mutation in any of these genes present in all probands.

4. Dominant (Other)

Prediction: One variant found in all probands and in no mothers. All genes related to DSD were looked at for mutations.

Gene list:

CBX2	DVL	WNT7B	BMP15
DHH	GATA4	WNT8A	NR0B1
SOX9	SOX10	WNT8B	NR0B2
FGF9	AXIN2	DVL1	NR5A1
FGR2	ERK1	DVL2	SOX3
SF1	ERK2	DVL3	SRY
MAP3K4	RSPO2	WNT8D	WT1
GSK3B	RSPO3	WNT9A	CDKN1C
AXIN1	RSPO4	WNT9B	GSK3B
DAX1	LGR4	WNT10-A	MAPK14
RAC1	LGR5	WNT1-B	SPRY2
FLNB	LGR6	WNT11	ROCK1
ROCK	WNT1	WNT16	CYP21A2
RHOA	WNT2	SFRP1	HSD3B2
DMRT1	WNT3	SFRP2	CYP11B1
MAP3K1	WNT4	FRZB	POR
FRAT1	WNT5A	SFRP4	CYP17A1
WNT4	WNT5B	SFRP5	STAR
RSPO1	WNT6	CTNNB1	CYP19A1
WWOX	WNT7A	APC2	PGD2
			FOXL2
			SOX8

Table 4.4 List of Genes studied
The results did not find a mutation in any of these genes present in all probands.

5. Recessive

Prediction: Variant found on both alleles in probands and only on one allele in mothers.

All genes were analyzed but the results did not find any convincing causal variant for Ovotesticular DSD.

CHAPTER FIVE

5.0 DISCUSSION

The primary objective of this study was to determine the genetic basis of 46XX Ovotesticular DSD among black South Africans. Whole genome analysis was performed on patients with 46XX Ovotesticular DSD and their biological parents to identify possible variants that may be responsible for the high prevalence of 46XX Ovotesticular DSD in this population. The clinical features of these patients were also analyzed as part of the study. Generally, the clinical features of the patients in this study was found to be consistent with those found in previous studies on black South Africans with Ovotesticular DSD and the clinical presentation again varied significantly with those found in patients from other parts of the world. Thus, this study also showed the uniqueness of Ovotesticular DSD among black South Africans

5.1: Karyotype

This study found that the most common karyotype for Ovotesticular DSD in South Africa is 46XX. 13 of the 15 patients (86.7%) reviewed were 46XX. The genotype of the remaining 2 patients was not documented. This is consistent with findings from other parts of South African. In 2008, Wiersma and Ramdial(33) found that 88% of patients with Ovotesticular DSD have a 46XX karyotype with the remaining patients having either a 46XY karyotype, a chimeric 46XX/46XY karyotype or a 46XX/XO mosaic pattern.[2] However, when compared to other countries, the karyotype of patients with ovotesticular DSD shows geographical variations. In Europe 40.5% of 74 cases were 46,XX karyotype and 21.0% of the patients in North America had chromosomal mosaicism(30).Krob et al also observed that the 46,XY karyotype is extremely rare (7%) and equally distributed through Asia, Europe and North America(30). The relatively high prevalence of 46XX karyotype in South African as compared to other parts of the world probably suggests a different cause of Ovotesticular DSD in South Africa.

5.2: Type of Gonad

This study found that the ovotestis is the most common, accounting for 63.3% of gonads in Ovotesticular DSD patients. This was followed by the ovary, which accounted for 26.7% and lastly, the testis, which accounted for 10%. Similarly, other South African studies concluded that the ovotestis accounts for more than 50% of the gonads in ovotesticular DSD patients. Ramsey et

al conducted a study in 1988 and found that ovotestes were found in 52.8% of patients while Wiesman and Ramdial found in 2008 that ovotestes were found in 54% of South Africans with ovotesticular DSD(22,33). Krob et al also found the most common gonad in patients with Ovotesticular DSD worldwide was the ovotestis, present in 44.4% of 568 gonads (30). Gonads with testicular tissue were more frequent on the right side of the body, while pure ovarian tissue was more common on the left both in South Africa and also in other parts of the world. (33,22,30).It is interesting to note that South African patients do not only have relatively higher percentages of ovotesticular DSD but as compared with other parts of the world where most of these ovotestis have a bipolar constitution, the most common constitution of ovotestis in South Africa is the admixed type as found by Weismar and Ramdial(33). This is also another observation that suggests the possibility of a different aetiology for Ovotesticular DSD in South Africa as compared to other parts of the world.

5.3 Distribution of gonad

The most common distribution is unilateral, with an ovary and ovotestes being present. This accounted for 53.3% of patients (8 out of 15 patients) with the majority of patients having an ovary on the left and an ovotestis on the right (7 out of 8 patients ie. 87.5%). This finding was consistent with the observation by Ramsay et al in their study which showed that in South Africa 51.4% of patients present with this unilateral distribution of gonads. Additionally, these studies found that the ovary was also most commonly found in the left pelvis(22). Vilain also stated that the distribution worldwide is 34% ovotestes/ovary, 29% ovotestes/ovotestes and 25% ovary/testes(32).

In this study, bilateral ovotestes are the second most common distribution, accounting for 26.7% of gonadal distributions. This is consistent with the observation by Vilian. Similarly, Ramsay and colleagues found that bilateral ovotestes was the second most common gonadal distribution in South Africa, accounting for 18.9% of gonadal distribution in their study (22).

Therefore, gonadal distribution in South Africa is generally consistent with that from other parts of the world.

5.4 Sex of Rearing

This study found that, in South Africa, patients with ovotesticular DSD were more commonly raised as female than males. 60% of the patients were raised as females while 40% of the patients were raised as males. This is consistent with the study by Ramsay et al, based in South Africa, which found 17 out of 33 patients (51.5%) to be female and 13 out of 33 patients (39.4%) to be male (3 patients' sex was ambivalent)(22).This shows a change in trend when compared with the observation by Van Niekerk in 1974 which showed that in the past, most of these patients were raised as males.(20). This change may have been an influence of the degree of virilization and the fact that that normal

ovarian tissue could be preserved in those being raised females. Hence, since the ovary is the second commonest gonad found in these children it is likely contributing significantly to the reversed trend for the chosen sex of rearing. The sex of rearing in South Africa now shows a different trend to that from other parts of the world where two thirds of patients are reared as males(39). This also shows that Ovotesticular DSD in South Africa presents with features which are different from those observed worldwide and thus may suggest a possible difference in etiological factors.

5.5: Findings at laparoscopy

This study also explored the presence and presentation of a uterus and fallopian tubes in patients with ovotesticular DSD. The majority of patients, (66.7%) presented with a uterus, 50% of which were abnormal or rudimentary while the remaining 50% were normal uterus. Furthermore, 46.7% of patients presented with fallopian tubes, the majority of which were normal in structure. This is consistent with findings that a uterus is usually (90%) present based on cases reported worldwide and as in Van Niekerk's sample(20). Sometimes the uterus is bicornuate or unicornuate. Similarly, in their series, also from South Africa, Wiersma and Randial reported that 58% of 125 patients had a "uterine structure"(33).The absence of a uterine horn often indicates ipsilateral testis or ovotestis. The fimbriated end of the fallopian tube is usually occluded ipsilateral to an ovotestis. The presence or absence of fallopian tubes or Wolffian derivatives reflects the ipsilateral gonad. A fallopian tube is invariably present ipsilateral to an ovary, whiles a vas deferens, epididymis, and, often, a seminal vesicle are usually present ipsilateral to a testis. A fallopian tube or Wolffian derivatives may be present on the side of an ovotestis, although most often a fallopian tube is present. Although fallopian tubes and Wolffian derivatives are usually not both present on the same side, even on the side of an ovotestis, this combination has been observed occasionally(36).

5.6 Genetic analysis for the etiology of 46XX Ovotesticular DSD.

Whole genome analysis was performed on patients with 46XX Ovotesticular DSD and their biological parents to identify possible variants that may be responsible for the high prevalence of 46XX Ovotesticular DSD in black South Africans. The study looked out for a variant in any of the genes known to promote formation of the testis. It was hypothesized that if a variant was found in any of these genes for all probands, then it will be the likely genetic aetiology for the formation of a testis in these 46XX individuals. The genes considered in this category were the SRY, RAC1, MAP3K4, AXIN1, SOX3, SOX8, SOX9, FGF9, FGFR2, SRY and GADD45Y genes. Although most of these genes especially duplications and triplications of SOX9 have been found in patients with 46XX sex reversal and some patients with 46XX Ovotesticular DSD from other parts of the world , none of these genes was found as a causal variant in any of the

probands. This is consistent with the findings of Padoa who also found no convincing role of SOX9 in the etiology of 46XX Ovotesticular DSD in South Africa.(129)

The study did not find any convincing evidence for haploinsufficiency of the genes RHOA, ROK, ERK1, ERK2, FRAT1, P38 which are known to cause down regulation of testes development. There was no convincing evidence of the role of genes known to cause DSD as the possible cause of 46XX Ovotesticular DSD in the patients for this study. This is consistent with similar studies for the molecular etiology of 46XX Ovotesticular DSD among South Africans which has so far been elusive (129).
This study has been consistent with other studies which show that Ovotesticular DSD in South Africa has different clinical presentation compared with those from other parts of the world. Almost all patients with Ovotesticular DSD in South Africa are 46XX and almost all the ovotestis are admixed with the majority of patients being raised as females. It is therefore not surprising that whereas genetic mutations such as duplication and triplication of SOX 9 has been found in patients with XX Ovotesticular DSD from other parts of the world, this study and similar South African studies have still not found any convincing genetic variant for this condition among South Africans.

It is possible that the unique features of 46XX Ovotesticular DSD among South Africans truly imply an alternate etiology in comparism to patients from other parts of the world. Although the full sequence of these genes was examined in this study, it is possible that there is a common etiologic factor in some aspect of the genome which has not been critically examined yet.

Limitation of the Study:

This study is limited by the small number of study participants. This is due to the rarity of Ovotesticular DSD and the refusal of some patients diagnosed with this disorder to consent to the study. The chances of finding a genetic etiology would have been enhanced with a larger sample size.

5.7 Conclusion:

The gene(s) involved in the etiology of XX Ovotesticular DSD have not yet been identified. A previous study on South African XX Ovotesticular DSD failed to implicate major environmental factors in the disorder(22). A simple single-gene explanation is unlikely if one considers that most cases are sporadic. (22,64)

Although others have found gene mutations such as duplication of SOX 9 in patients with Ovotesticular DSD from other parts of the world, this study and similar studies from South Africa have failed to demonstrate any gene mutations in Ovotesticular DSD in South Africa. However, this study has once again confirmed the strikingly higher frequency of 46XX Ovotesticular DSD in South Africa, the different pattern of gonadal distribution and the high frequency of female sex rearing as compared to other parts of the world. These unique differences suggest a possible different etiology to Ovotesticular DSD in South Africa. Although the full sequence of these genes was examined in this study, it is possible that there is a common etiologic factor in some aspect of the genome which has not been critically examined yet.
Finding the molecular etiology may lie in doing a more discreet analysis of the entire genome.

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