Aus dem Institut für Experimentelle Pädiatrische Endokrinologie
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DISSERTATION

Untersuchung des Steroidogenic Factor 1 kodierenden Gens *NR5A1* in einer Kohorte von 50 ägyptischen Patienten mit 46,XY Störungen der Geschlechtsentwicklung

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von

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Abstract

Untersuchung des Steroidogenic Factor 1 kodierenden Gens *NR5A1* in einer Kohorte von 50 ägyptischen Patienten mit 46,XY Störungen der Geschlechtsentwicklung

Einleitung: Steroidogenic Factor 1 (SF-1) ist ein zentraler Transkriptionsregulator von Genen, die eine vielseitige Rolle in der Hypothalamus-Hypophysen-Gonaden-Achse spielen und die Entwicklung und Funktion der Gonaden beeinflussen. In den letzten Jahren wurde festgestellt, das SF-1-Mutationen eine häufige Ursache von 46,XY Störungen der Geschlechtsentwicklung („disorders of sex development", DSD) beim Menschen sind. Ziel der Arbeit ist die Untersuchung der Häufigkeit von SF-1 Mutationen in einer ägyptischen XY DSD Kohorte. Bisher wurde das Vorkommen von SF-1-Mutationen in anderen Kohorten im Mittleren Osten nicht untersucht.

Methodik: Molekulargenetische Untersuchung des für den Transkriptionsfaktor SF-1 kodierenden Gens *NR5A1* im Rahmen einer Fall-Kontroll-Studie. Fünfzig ägyptische XY DSD Patienten mit folgendem phänotypischen Spektrum wurden untersucht: äußerlich normal weibliches Genitale mit/ohne Uterus (n = 8), intersexuelles Genitale ohne Uterus (n = 5), Hodenatrophie (n = 10), Hypospadie (n = 23), und isolierter hypoplastischer Phallus (n = 4). Bei keinem Patienten wurde eine Nebenniereninsuffizienz beobachtet.

Ergebnisse: Nachweis von 2 neuen heterozygoten Mutationen im kodierenden Bereich von *NR5A1*. Die p.Glu121AlafsX25 Mutation führt zu einem stark trunkierten SF-1 Protein. Die p.Arg62Cys Mutation liegt in der DNA-bindenden Zinkfinger-Region. Funktionelle Untersuchungen der p.Arg62Cys Mutation in-vitro zeigten eine deutlich reduzierte transkriptionelle Aktivierung. Die p.Ala154Thr Mutation zeigte hingegen keine reduzierte transkriptionelle Aktivierung. Siebzehn Patienten (34%) waren Träger des bekannten p.Gly146Ala Polymorphismus.

Schlussfolgerung: Es wurden zwei neue funktionell relevante SF-1-Mutationen bei 2 von 23 Patienten mit Hypospadie (8,5%) gefunden. Dieses Ergebnis entspricht früheren europäischen Studien, die in bis zu 10% der XY DSD-Patienten *NR5A1* Mutation beschrieben haben. Diese Studie unterstreicht die Bedeutung kollaborativer Untersuchungen, um mehr Kenntnisse über das Auftreten von *NR5A1* Mutationen bei Patienten mit DSD und unterschiedlichem genetischen Hintergrund zu gewinnen.

Abstract

Es sollten regelmäßige Kontrollen der Gonadenfunktion und eine frühe Kryokonservierung von Spermien bei diesen Patienten in Erwägung gezogen werden, da das Risiko einer zunehmenden Gonadeninsuffizienz bei Männern mit SF-1 Mutationen im Jugend- und Erwachsenenalter sehr hoch ist. Es ist empfehlenswert, das SF-1-Gen bei XY DSD Patienten mit schweren und leichteren Androgenisierungsstörungen und Gonadendysgenesie zu untersuchen. Weitere Verlaufskontrollen und Studien und sind erforderlich, um die Langzeitfunktion der Testes und das Tumorrisiko bei Patienten mit SF-1-Mutationen einschätzen zu können.

Abstract

Molecular genetic analysis of *NR5A1* the gene coding for steroidogenic factor 1 in Egyptian patients with 46,XY disorders of sex development

Background: Steroidogenic factor 1 (SF-1) is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-gonadal axis. Recently, SF-1 mutations have been emerging as a frequent cause of 46,XY DSD in humans. The objective of the work is to investigate SF-1 mutations as a cause of 46,XY DSD in Egypt. SF-1 mutations have not yet been studied in other oriental cohorts.

Methods: Molecular genetic analysis of *NR5A1* gene coding for SF-1 in a case-control study. Fifty Egyptian XY DSD patients (without adrenal insufficiency) with a phenotypic spectrum from complete female external genitalia with/without uterus (n=8) to ambiguous genitalia without uterus (n=5), vanishing/atrophic testes (n=10), hypospadias (n=23) and isolated hypoplastic phallus (n=4).

Results: Two novel heterozygous mutations of the coding region in patients with hypospadias were detected. p.Glu121AlafsX25 results in a severely truncated protein and p.Arg62Cys lies in the DNA-binding zinc finger region of the SF1 protein. Functional studies showed that p.Arg62Cys mutation resulted in a significant reduction of SF-1 transcriptional activity, while p.Ala154Thr showed no reduced activity. A total of 17 patients (34%) harbored the known p.Gly146Ala polymorphism.

Conclusion: This study detected two novel SF-1 mutations showing impaired function in 2 of 23 Egyptian XY DSD patients with hypospadias (8.5%). The study corresponds to the results of European studies showing that up to 10% of XY DSD patients harbour a mutation in *NR5A1*. It highlights the importance of collaborative efforts in increasing the knowledge about the occurrence of SF-1 in patients with DSD and different genetic background. Regular monitoring of gonadal function in adolescence and adulthood in males with *NR5A1* mutations is strongly recommended, as it has been previously reported that *NR5A1* mutations can cause a progressive disease with deterioration of gonadal function with advancing age. Subsequently, early cryoconservation of detected sperms in attempt to preserve future chances of fertility should be considered. Because of the high prevalence of *NR5A1* mutations in XY DSD patients with severe and milder

forms of underandrogenization due to gonadal dysgenesis, it is recommended to screen for *NR5A1* mutations in this subset of patients. Patients harboring mutations should be followed up as long-term outcome of gonadal function and gonadal malignancy risk are unclear.

1 Introduction

1.1 Disorders of sex development

“Is it a boy or a girl?” a question that always arises at the birth of a baby. Everywhere all over the world, it is usually expected of one of the parents to shout out the sex of the baby to the relatives waiting in anticipation. The answer is typically easy and straightforward, however for a few it is a challenge and a riddle that urgently awaits the solution. When neither typical male nor female external genitalia can be identified, it is usually referred to as ambiguous genitalia. With atypical genitalia, assigning a sex to the infant is most difficult and thus logically postponed [1]. These ‘congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical’ are termed disorders of sex development (DSD) [2].

In humans, sex development is a complex process with many intercommunicating factors which, if disturbed at crucial points, have a major biological, psychological, social and sexual impact on the affected person. It consists broadly of three main processes; chromosomal establishment during fertilization, gonadal determination (ovary or testis) and sex differentiation of internal and external genitalia [3].

DSD constitute a spectrum of genitourinary tract and endocrine reproductive system disorders [2]. Diagnosing and treating children with DSD is a quite difficult task even in the most advanced care centres [1]. The European society for paediatric endocrinology (ESPE) and the Lawson Wilkins paediatric endocrine society (LWPES) devised a consensus statement in 2006 proposing a new nomenclature and DSD classification system as well as outlining the recommendations for the management of disorders of sex development (*Table 1*) [2]. Although management guidelines have been made available, the most suitable method remains the personal individual approach with respect to the standard guidelines [1]. According to the new DSD classification, DSD are categorized according to the karyotype and the pathogenesis into sex chromosome DSD, 46 XY DSD and 46 XX DSD (*Table 1*) [2].

Table 1: Classification of DSD as proposed by the DSD consensus group 2006 [2]

Sex chromosome DSD	46,XY DSD	46,XX DSD
A. 45,X (Turner syndrome and variants)	**A. Disorders of gonadal (testicular) development** 1.Complete gonadal dysgenesis (Swyer syndrome) 2. Partial gonadal dysgenesis 3. Gonadal regression 4. Ovotesticular DSD	**A. Disorders of gonadal (ovarian) development** 1. Ovotesticular DSD 2. Testicular DSD (eg, SRY+, dup SOX9) 3. Gonadal dysgenesis
B. 47,XXY (Klinefelter syndrome and variants)	**B. Disorders in androgen synthesis or action** 1. Androgen biosynthesis defect (eg. 17-hydroxysteroid dehydrogenase deficiency, 5α reductase deficiency, StAR mutations 2. Defect in androgen action (eg, CAIS, PAIS) 3. LH receptor defects (eg, Leydig cell hypoplasia, aplasia) 4. Disorders of AMH and AMH receptor (persistent mullerian duct syndrome)	**B. Androgen excess** 1. Fetal (eg, 21-hydroxylase deficiency, 11-hydroxylase deficiency) 2. Fetoplacental (aromatase deficiency, POR) 3. Maternal (luteoma, exogenous, etc)
C. 45,X/46,XY (mixed gonadal dysgenesis, ovotesticular DSD)	**C. Other** (eg, severe hypospadias, cloacal extrophy)	**C. Other** (eg, cloacal extrophy, vaginal atresia, MURCS, other syndromes)
D. 46,XX/46,XY (chimeric, ovotesticular DSD)		

The most common cause of genital ambiguity is known to be congenital adrenal hyperplasia (CAH), followed by mixed gonadal dysgenesis and 46,XY DSD. The majority of virilised 46,XX infants will have CAH, a recent study detected CAH in 86% of 46,XX DSD patients [4]. In contrast, only a minority of 46,XY children with DSD will receive a definitive diagnosis [5, 6], a most recent study could detect a genetic diagnosis in 35% of 46,XY DSD patients using next generation whole-exome sequencing [7].

46,XY DSD includes XY partial or complete gonadal dysgenesis, androgen synthesis and action defects and unknown causes resulting in hypospadias, which all result in undermasculinization of an XY male. Some of these phenotypes, such as 46,XY gonadal dysgenesis are relatively rare, whereas the 46,XY DSD-related phenotype hypospadias (*Figure 1*), a congenital defect affecting normal development of the male urogenital external tract in which the urethral orifice of the penis is positioned ventrally

[8], is a relatively common congenital anomaly with an estimated incidence of 1:200–1:300 live births [9]. A recent systematic review on hypospadias [10] has shown that figures on the birth prevalence of hypospadias vary considerably across countries, ranging from 4 to 43 cases per 10 000 births [11, 12]. In Egypt, the incidence of hypospadias is 13:10,000 (1:769) live births making it a common anomaly among Egyptians [13]. The etiology of hypospadias could be gonadal dysgenesis, androgen synthesis defect, early developmental gene mutations, but in the majority of cases the cause remains unknown.

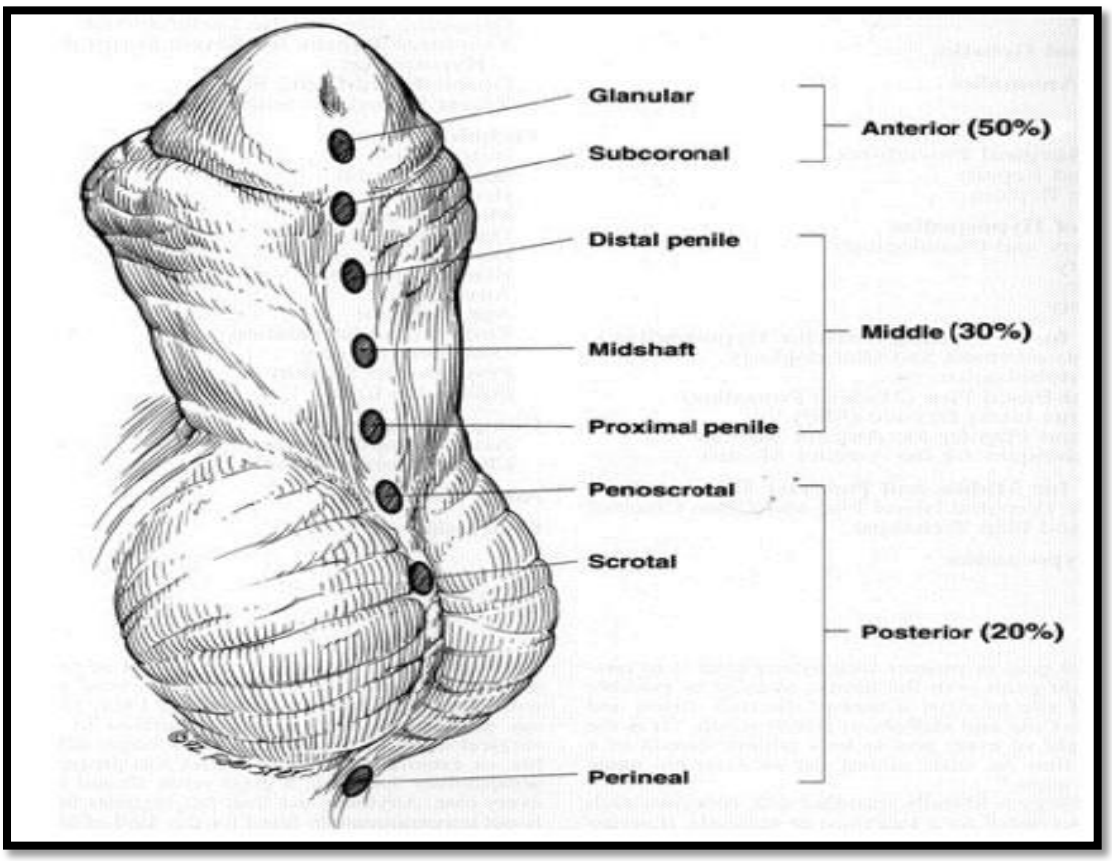

Figure 1: Classification of hypospadias based on anatomic location of the urethral meatus. Anterior, or distal, hypospadias is the most commonly encountered variant. Figure adapted from Kraft et al. [14] with permission.

1.2 Role of steroidogenic factor 1 (SF-1) in sexual development

So far, mutations of SF-1 and SRY are the most common causes (10%) in partial XY gonadal dysgenesis and complete XY gonadal dysgenesis. Steroidogenic factor 1 (SF-1, *NR5A1*), a member of the nuclear receptor superfamily, was first described as a

major regulator of cholesterol metabolism in steroidogenic cells. It stimulates the expression of nearly all factors involved in cholesterol mobilization and steroid hormone biosynthesis [15]. Moreover, it is a transcriptional regulator of genes involved in the hypothalamic-pituitary-gonadal axis and in the adrenal cortex. But mainly in the developing human gonad, SF-1 participates with several other transcription factors (WT1, DAX1, SRY and SOX9) in determination of the testis (*Figure 2*). Hence SF-1 has a pivotal role in sex determination and differentiation. In Sertoli cells, SF-1 regulates the expression of anti-Müllerian hormone, which leads to regression of the progenitors of the oviducts, uterus and upper vagina [16]. In Leydig cells, SF-1 regulates transcription of various enzymes involved in steroidogenesis and testosterone biosynthesis, allowing virilization of the male fetus [17]. In the developing ovary, SF-1 transcript levels fall during embryogenesis but are expressed in the granulosa and theca cells of the adult ovary at the onset of folliculogenesis [18]. Finally, SF-1 also plays an important role in the development of the ventromedial hypothalamus and pituitary gonadotrophs [19].

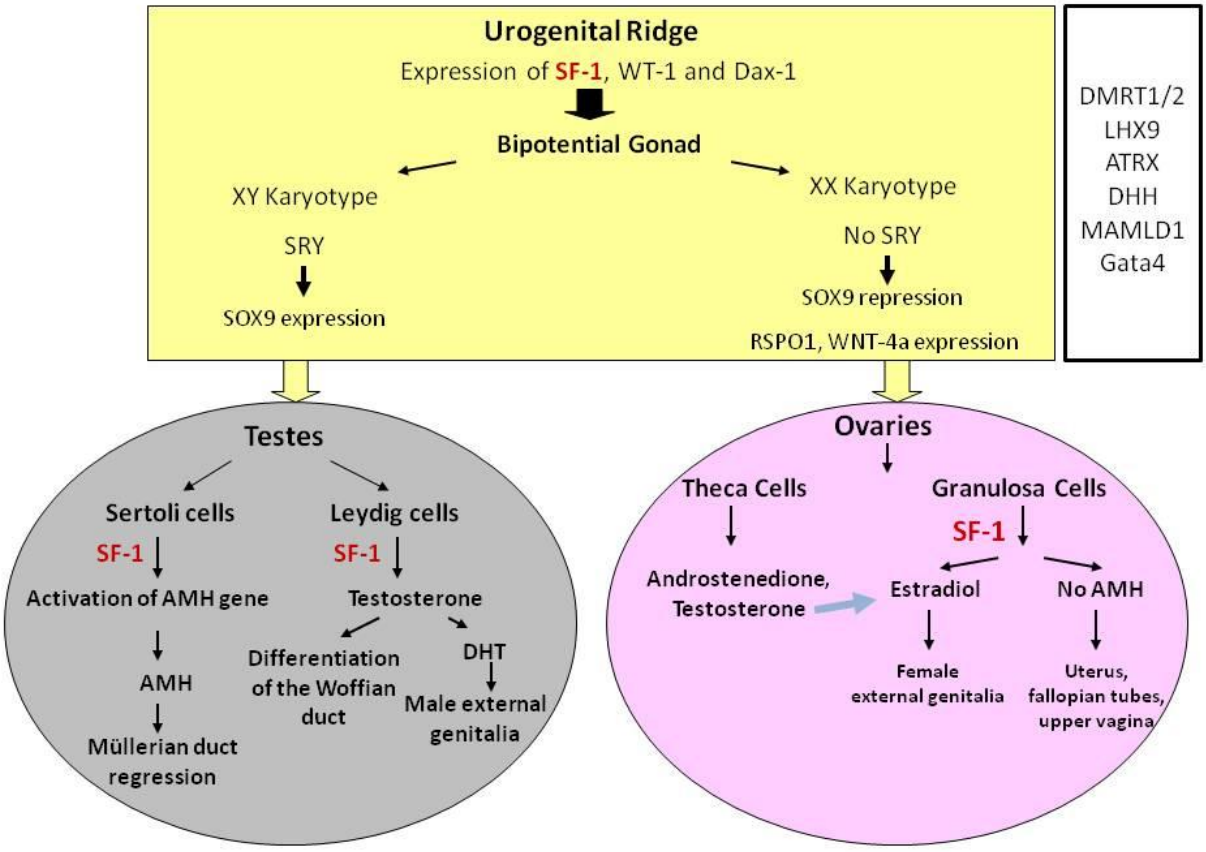

Figure 2: Expression of SF-1 in intermediate mesoderm, genital ridge and gonads during embryonic life.

SF-1 was first identified as a transcription factor of genes encoding the cytochrome P450 steroid hydroxylases [20]. Further discoveries of SF-1 roles were determined by Luo et al. in 1994 when they generated sf-1 knockout mice (sf1-/-) [21]. These mice lacked adrenal glands and gonads (*Figure 3*) and were severely deficient in corticosterone, supporting adrenocortical insufficiency. Male sf-1 null mice also showed complete sex reversal. In both male and female (sf1-/-) mice, oviducts formed in a normal female pattern as well as normal uteri and vagina [21].

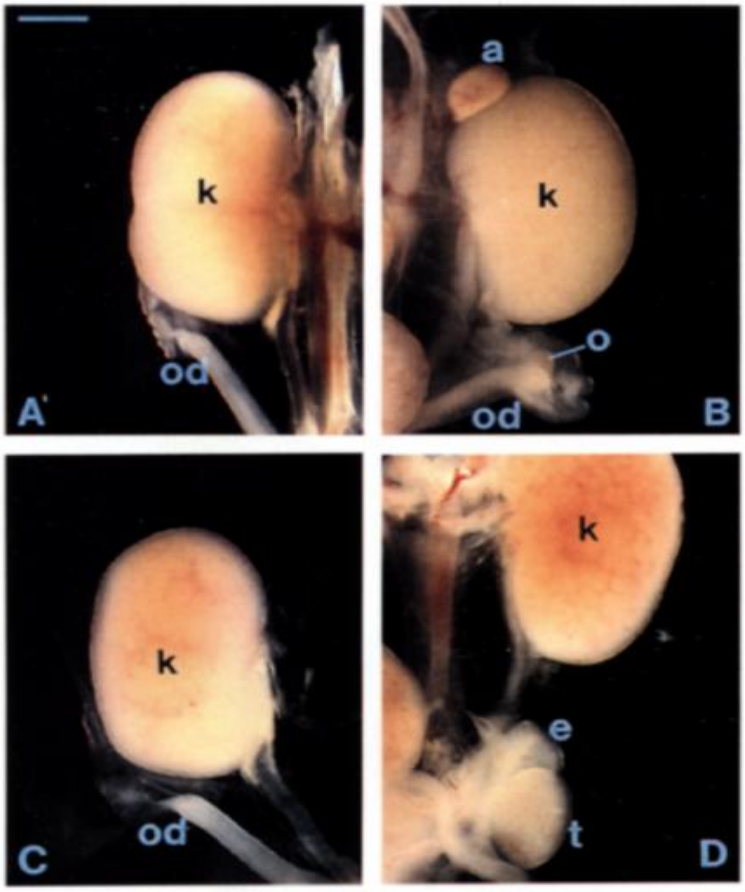

Figure 3: Newborn sf1 (Ftz-Ff) null mice lack adrenal glands and have female sexual phenotype. Ffz-F1 null pups and wild-type +/+ or +/- littermates were sacrificed, and the genitourinary systems were isolated by dissection. Sexual genotypes were determined by PCR analysis with Sry primers, as described in Experimental Procedures. (A) Ffz-F1 null female. (B) wildtype female. (C) Ffz-f 1 null male. (D) wild-type male. Scale bar, 1 mm. Abbreviations: k, kidney; a, adrenal; o, ovary; t, testis; e, epididymis; od, oviduct. - Figure adapted from [21] with permission.

Subsequent studies on sites of SF-1 expression revealed high expression in steroidogenic tissues of gonads and adrenal cortex [22] from early embryonic stages into adult life as well as pituitary gonadotropes and ventromedial hypothalamic nucleus

[19, 23, 24]. Multiple genes were identified as target genes of SF-1 in the different expression sites of SF-1; StAR [25, 26], CYP11A1, CYP17, LHR in Leydig cells of the testis; SRY in pre Sertoli cells of the testis [27], SOX9 [28], MIS (AMH), FSHR, CYP11A1, CYP17, INHA, Oxytocin in the ovary; StAR, CYP11A1 in the adrenal cortex as well as other important genes for adrenocortical function such as SOAT1 among several novel genes very recently identified [29] (*Table 2*).

Table 2: Overview of selected NR5A1 target genes [30]

Adrenal cortex	Zona glomerulosa	CYP11B2, CYP21, DAX-1 (NR0B1), SHP (NR0B2), CYP11A1, CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
	Zona fasciculata	CYP11B1, CYP21, DAX-1 (NR0B1), SHP (NR0B2), MC2R (ACTH-R), AKR1B7, CYP11A1,CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
	Zona reticularis	CYP11B1, CYP21, DAX-1 (NR0B1), SHP (NR0B2), MC2R (ACTH-R), SULT2A1, CYP11A1, CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
Ovary	Theca cells	alpha-Inhibin, DAX-1 (NR0B1), CYP11A1, CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
	Granulosa cells	CYP19, MIS (AMH), CYP11A1, CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
	Corpus luteum	CYP19, Oxytocin, CYP11A1, CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
Testis	Leydig cells	CYP19, INSL3, PRL-R, DAX-1 (NR0B1), SRY, Vanin-1, HMG-CoA synthase, CYP11A1, CYP17, 3ß-HSD, StAR, SCP-2, SR-BI (HDL-R), NP-C1
	Sertoli cells	CYP19, MIS (AMH), FSH-R, SRY, Sox-9, Vanin-1
Pituitary	Gonadotropes	LHß, FSHß, GnRH-R, alpha-GSU, nNOS, alpha-Inhibin
Hypothalamus	VMH	NMDA-R, BDNF

Further SF-1 studies on male gonad-specific sf-1 knockout mice showed hypoplastic testes and internal genital structures (seminal vesicles, epididymides and vasa deferentia), undescended testes and infertility (*Figure 4*). Female gonad-specific sf1−/− mice were infertile due to the absence of corpora lutea in the ovaries and the decreased number of ovarian follicles [31]. *In vitro* SF-1 interacts with SOX-9 and GATA-4 to prevent the differentiation of female internal genitalia by stimulating the expression of the Müllerian inhibitory substance (MIS) necessary for regression of the Müllerian ducts [16, 32, 33].

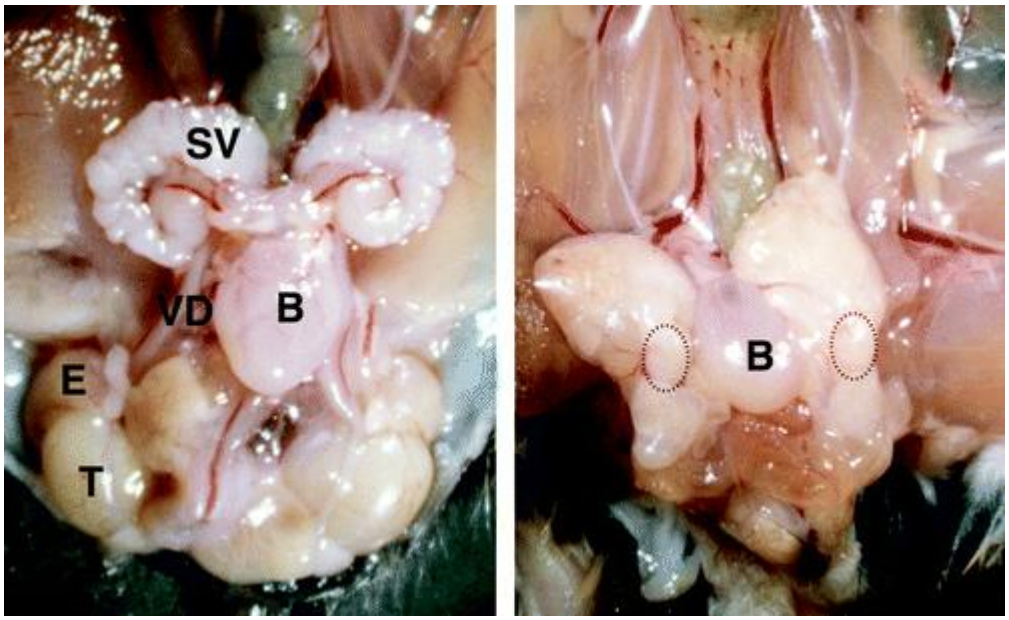

Figure 4: Structures of the testes and internal genitalia of gonad-specific SF-1 KO mice
Adult WT and gonad-specific SF-1 KO males were killed and their internal structures were displayed. *Left*, WT male. *Right*, SF-1 KO male. Testes in the gonad-specific SF-1 KO male are indicated by the *dotted ovals*. T, Testis; SV, seminal vesicle; E, epididymis; B, bladder; U, ureter; VD, vas deferens. Figure adapted from Jeyasuria et al. [31] with permission.

1.3 Molecular aspects of SF-1 (*NR5A1)*

SF-1 gene's official symbol is *NR5A1* (nuclear receptor subfamily 5, group A, member 1), HGNC: 7983, NCBI Reference Sequence: NM_004959.4, OMIM: 184757, Ensembl transcript version ENST00000373588.4. It is also known as AD4BP, FTZF1 (Drosophila) or Ftz-F1 (Mouse) The nuclear receptor (NR) steroidogenic factor 1 was first isolated in the early 1990s by two research teams (Parker and Morohashi) searching for transcription factors that interacted with promoter elements in steroid hydroxylase genes (CYPs) [30]. The name SF-1 was given by Parker et al. to the mammalian homolog, whereas Morohashi et al. called the protein adrenal 4-binding protein (Ad4BP) based on its interaction with the Ad4 element in the bovine *CYP11B1* promoter [34]. It is located on the long arm of chromosome 9 in position 33.3 (9q33.3):127,243,516 - 127,269,709, 26,193 bps long, consisting of 7 exons; exon 1 non-coding and exons 2-7 code for 461 amino acids with a transcript length of 3,104 bps (*Figure 5,* [35]).

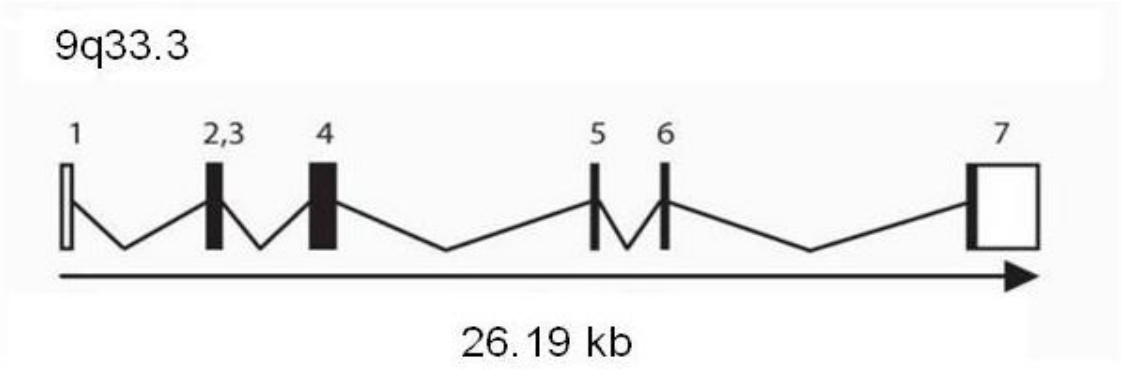

Figure 5: An overview of the genomic structure of NR5A1 (encoding SF-1). Figure adapted from Lin et al. [35] with permission.

NR5A1 harbours the classic structural features of a nuclear receptor; a DNA-binding domain (DBD) with two Cys2–Cys2 zinc fingers, a flexible hinge region, a ligand-binding domain (LBD), and two activation function domains; AF-1 and AF-2 (*Figure 6*) [30]. The FTZ-F1 box is an extension of the DBD which is important for DNA anchoring, and is present in other members of the NR5A subfamily. The A- and T-boxes in the FTZ-F1 domain as well as the P-box in the DBD stabilize the interaction with DNA and define the specificity of the DNA binding [36]. The AF-1 domain is positioned in the hinge region while the AF-2 domain is located at the C-terminal of the LBD and is essential for transcriptional activity and cofactor interactions *(Figure 6)* [37]. The hinge region is important for post-transcriptional/translational modifications. [30].

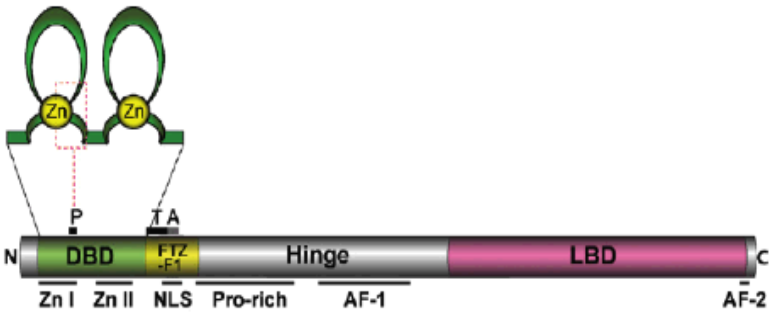

Figure 6: A schematic overview of SF-1.
SF-1 contains the DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD). SF-1 also contains an FTZ-F1 domain specific for the NR5A subclass. The two zinc fingers (Zn I, Zn II) of the DBD are highlighted on top. The P-box within zinc finger I is indicated by a red dashed square. NLS: nuclear localization signal, Pro-rich: proline-rich region, Ftz-F1: Fushi-tarazu factor-1 box, P/T/A: “DNA-binding boxes”. - Figure adapted from Hoivik et al. [30] with permission.

1.4 SF-1 mutations and disorders of sex development

To date, about 60 different *NR5A1* mutations have been reported in humans with disorders of sex development (DSD). *NR5A1* mutations were first described in two 46,XY patients with complete gonadal dysgenesis and primary adrenal failure. The patients displayed female external genitalia and Müllerian structures [38, 39]. The first was a de novo heterozygous mutation (p.G35E) in the first zinc finger of the DNA-binding domain severely affecting SF-1 function [38]. The second was a recessively inherited homozygous change (p.R92Q) affecting the ‘‘A’’ box of SF-1 which reduces the functional activity of SF-1 i.e. causing partial loss of function [39]. So far, only one additional *NR5A1* mutation has also been reported in a 46,XX female with primary adrenal failure [40].

Subsequently, heterozygous *NR5A1* mutations were identified in patients with 46,XY DSD without adrenal insufficiency. In 2004, the first 46,XY patients with ambiguous genitalia and partial gonadal dysgenesis, due to *NR5A1* mutations, without adrenal insufficiency were described [41-43]. Since then, *NR5A1* mutations were identified in an increasing number of 46,XY DSD patients, the majority of whom shared a phenotype of ambiguous genitalia at birth, partial gonadal dysgenesis and absent or rudimentary Müllerian structures. SF-1 mutations have been reported in 6.5-15% of 46,XY DSD severe 46,XY partial gonadal dysgenesis cohorts [44-47] as well as in 3-5% of hypospadias cases due to moderate 46, XY partial gonadal dysgenesis [44, 45]. Furthermore, SF-1 also plays a role in ovarian development and function. *NR5A1* mutations could be identified in a few cases of primary ovarian insufficiency and premature ovarian failure [48].

So far, there is no apparent genotype-phenotype correlation in patients with *NR5A1* mutations. In 46,XY patients, the phenotypic spectrum has been extended involving not only ambiguous genitalia with partial gonadal dysgenesis, but also hypospadias and cryptorchidism, which are likely to be caused by a milder form of partial gonadal dysgenesis [45, 49], as well as in association with vanishing testis syndrome, micropenis, or male infertility [50-53].

1.5 46,XY DSD in Egypt

In Egypt, the incidence of ambiguous genitalia is 1:3,000 compared to 1:5,000 in Germany [54, 55]. This can be attributed in part to the high rate of consanguineous marriages in Egypt which during the past 40 years ranged between 29 and 39% [16, 18, 55-58]. First cousin marriages constituted 15.6%, second cousin marriages 7% and further relative marriages 7.1% [59]. A study on 10,000 Egyptian couples revealed a consanguinity rate of 35.3% in 2011, especially first cousin marriages [60].

46, XY DSD constitute the majority (65.9%) of DSD cases in Egypt (*Figure 7*) [61]. Available studies on differential diagnosis of DSD in Egypt are limited. A study including 208 patients with ambiguous genitalia revealed 65.9% of the cases to be due to 46,XY DSD, 28% 46,XX DSD and around 10% could not be followed up and hence not diagnosed [61]. In Egypt, androgen insensitivity syndrome (AIS) and 5 alpha-reductase II deficiency are the two most frequent 46,XY DSD etiologies due to high consanguinity [62, 63]. They constitute 64% among all reported cases [63]. This has never been confirmed at a molecular level, however. The high cost of molecular genetic diagnosis remains a barrier. Differential diagnosis of different 46,XY DSD in Egypt based on a molecular diagnosis remains unavailable to date.

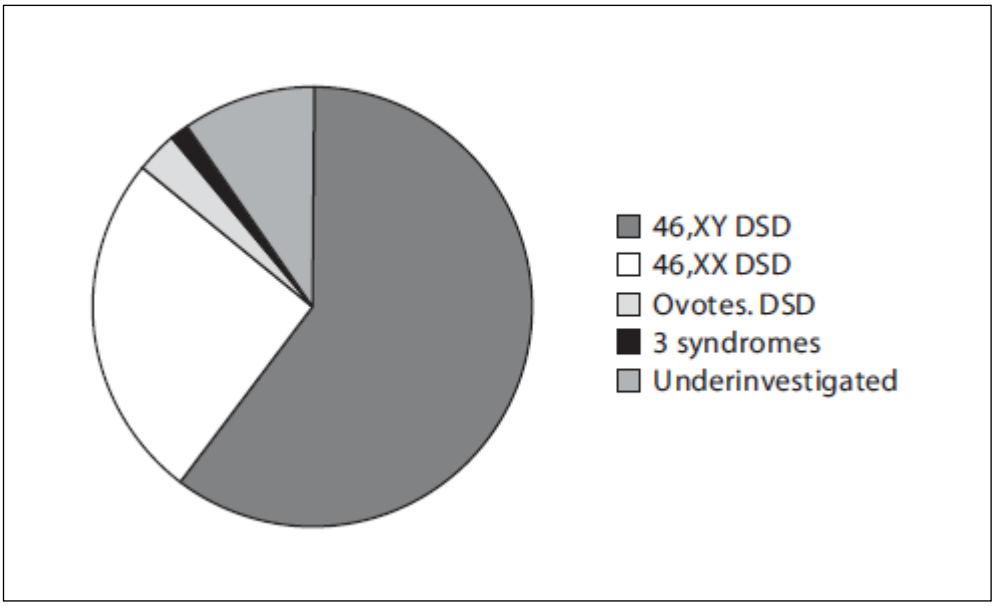

Figure 7: Differential diagnosis of DSD in Egypt.
A study including 208 Egyptian patients with ambiguous genitalia, revealing 46,XY DSD to be the commonest diagnosis. Figure adapted from Mazen et al. [61] with permission.

1.6 Objective of the work

The aim of this work was to investigate *NR5A1* mutations as a cause of 46,XY DSD in Egypt, as recently SF-1 mutations have been emerging as a frequent cause of 46,XY DSD in Germany and in Europe, ranging from 6.5 - to15% [44-46]. This study involves a large cohort of Egyptian patients with mild to severe forms of 46,XY DSD. So far, SF-1 mutations have not been studied in other oriental cohorts.

2 Patients, materials and methods

2.1 Patients

A cohort of fifty Egyptian 46,XY DSD patients were recruited from the pediatric endocrinology and the clinical genetics clinics of the National Research Centre (NRC) in Cairo, Egypt. Patients' chronological ages ranged from 2 months to 33 years at first presentation. Phenotype spectrum included complete female external genitalia with/without uterus (n=8), ambiguous genitalia without uterus (n=5), vanishing/atrophic testes (n=10), hypospadias (n=23) and isolated hypoplastic phallus (n=4) (*Table 3*). Patients with syndromic forms of gonadal dysgenesis or chromosomal abnormalities were excluded. Patients with a history of exposure to environmental xenoestrogens (such as herbicides, pesticides, polychlorinated biphenyls, plasticizers and polystyrenes), which mimic estrogens, or environmental antiandrogens (such as polyaromatic hydrocarbons, linuron, vinclozolin, and pp'DDE) that disturb endocrine balance and cause demasculinizing effects in the male fetus, were also excluded. Among the 50 patients, 39 were reared as male, 11 as females and 1 was reared as female at birth but at 2 years reared as male. Informed consent was obtained from all patients or their parents to perform molecular analysis. Work up included karyotype, pelviabdominal ultrasound and hormonal profile.

Table 3: Phenotypes of the cohort of XY DSD patients

Genitalia		**Gonads**
Female external genitalia *n=8*	with uterus *n=1*	No testicular/ovarian tissue
	no uterus *n=7*	Undescended testes
Ambiguous genitalia no uterus *n=5*		No testicular/ovarian tissue *n=1*
		Undescended testes *n=4*
Hypospadias *n=23*	hypoplastic phallus *n=16*	Undescended testes *n=10*
		Normally descended testes *n=6*
	normal sized phallus *n=7*	Undescended testes *n=4*
		Normally descended testes *n=3*
Hypoplastic phallus *n=4*		Undescended testes *n=1*
		Normally descended testes *n=3*
Normal phallus *n=10*		Bilateral atrophic testes *n=9*
		Unilateral atrophic testis *n=1*

2.2 Materials

2.2.1 Equipment

The following equipment were used throughout this study

Device	Manufacturer
Biophotometer 6131	Eppendorf, Hamburg
Centrifuge 5417R	Eppendorf, Hamburg
Gel electrophoresis chamber	Bio-Rad-System, Hercules CA
Gene Flash Gel documentation system	Syngene Bio Imaging
GeneAmp PCR System 9700	Perkin Elmer, Shelton CT
Horizontal electrophoresis system	Whatman, Biometra, Goettingen
Icemaker	Scotsman
Maxwell®16 DNA Instrument	Promega, Mannheim
Photometer	Eppendorf, Hamburg
Pipettes	Eppendorf, Hamburg
Protein gel electrophoresis chamber	Bio-Rad-System, Hercules CA
Sequencer (ABI 310)	Perkin Elmer, Shelton CT
Suction	Hartenstein, Würzburg
Thermal cycler Mastercycler® ep gradient S	Eppendorf, Hamburg
Thermomixer compact	Eppendorf, Hamburg
UNO-Thermoblock	Biometra
Video graphic printer UP-897 MD	Sony

2.2.2 Chemicals and substances

The following chemicals and substances were using throughout this study

Manufacturer	Chemicals and substances
Applied Biosystems	BigDye Terminator v3.1 5x Sequencing Buffer
Bioline	Easy Ladder
Fermentas	dNTPs
Fluka	Betain, sodium
Invitrogen	Agarose
Merck	Ethidiumbromide, HPLC-H2O, Tris-borate, sodium acetate
Roche	Ethanol
Roth	NaCl
Sigma	Magnesium chloride (MgCl2), EDTA

2.2.3 Enzymes

The following enzymes were used throughout this study

Exonuclease I (Exo I)	New England Bio Labs
Shrimp Alkaline Phosphatase (SAP)	Affymetrix – USB products
TAQ-Polymerase and buffer	Bioline, Berlin

2.2.4 Kits

The following kits were used throughout this study

BigDye® Terminator v3.1 Cycle Sequencing Kit

DNA IQ™ Casework Pro Kit for Maxwell® 16

2.2.5 SF-1 primers (DNA oligonucleotides)

Primers were designed using NCBI/ Primer-BLAST website tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), and were generated by the Sigma-Aldrich company.

SF-1 2/3 (492 bp)
- Forward primer 5´- CAGAGAGGGGATTACGCGAC - 3’
- Reverse primer 5´- CGGTTCTCTTGCAGCGACTG - 3’

SF-1 4A (400 bp)
- Forward primer 5’- TTGTTTGGAAAGGATCTGTGG - 3’
- Reverse primer 5’ - AAGGCAGGGTAGAGGTAGCC - 3’

SF-1 4B (600 bp)
- Forward primer 5’ - TGGCTTCAAGCTGGAGACAG - 3’
- Reverse primer 5’ - AAAGGACAGTCGGGCTAAGG - 3’

SF-1 5 (590 bp)
- Forward primer 5´- CTTTGATAGCCTAGACATCTG - 3’
- Reverse primer 5´- CAACTCCAGTGTGTTATTTCC - 3’

SF-1 6 (571 bp)
- Forward primer 5´- CTGTGCAGATGTTACCCGTG - 3’
- Reverse primer 5´- CCCTAGATCCAGGGATTAGAG - 3’

SF-1 7A (340 bp)
- Forward primer 5´- TGGTGGCAGCAATGCCCATGTCT - 3’
- Reverse primer 5´- TGTTGCGGGGCATCTCGTTGC - 3’

SF-1 7B (291 bp)
- Forward primer 5´- TGCTTGACTACACCCTGTGC - 3’
- Reverse primer 5´- TGGGCATCAGAAAATGAACC - 3’

2.3 Methods

2.3.1 Overview

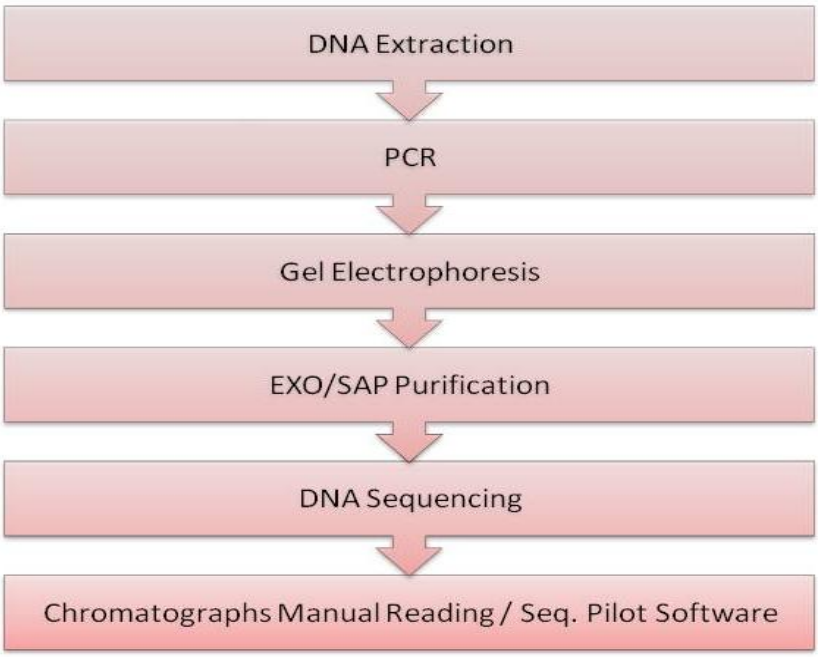

Figure 8: Steps of molecular analysis of SF-1 (NR5A1) gene

This is a case-control study of 50 46,XY DSD Egyptian patients versus 100 healthy Egyptian controls. Genomic DNA was extracted from peripheral blood leukocytes out of dried blood spots on filter cards (*see 2.3.2*). The entire coding region (exons 2–7) and splice sites of the gene encoding SF-1 (*NR5A1*) were PCR amplified (*see 2.3.3*). DNA was separated using gel electrophoresis and the quality of the PCR product was controlled and documented using Gene Flash Gel documentation system (*see 2.3.4*). PCR products were purified using exonuclease 1 and shrimp alkaline phosphatase (*see 2.3.5*) and sequenced using a V3 kit and 3130x analyzer (*see 2.3.6 and 2.3.7*). The chromatograms were interpreted using the conventional manual reading method and the SeqPilot software (JSI medical system GmbH, Kippenheim, Germany). DNA mutation numbering is based on GenBank reference DNA sequence NM_004959.3, with the A of the ATG initiation codon designated 1 (www.hgvs.org/mutnomen). *NR5A1* gene sequence Ensembl ENSG00000136931. DNA from one hundred healthy Egyptian

controls (200 alleles) was analyzed for detected mutations and single nucleotide polymorphisms.

2.3.1.1 DNA extraction

DNA was extracted from dried blood samples on filter cards using Maxwell 16 Automated DNA Purification instrument by DNA IQ™ Casework Pro Kit for Maxwell® 16. The lysis buffer was prepared by adding 5µl of 1M DTT to 500µl of lysis buffer and mixing by inverting several times. The filter card sample (recommended amount 15-50mm2) was placed in a 1.5ml microtube (Cat.# V1231), and 500µl of prepared lysis buffer (containing DTT) was added. Then it was briefly vortexed with closed lid and heated at 70°C for 30 minutes. After the 70°C incubation, the sample was briefly vortexed to recover any evaporated liquid on the sides of the tube. A fresh 1.5ml microtube was labelled and a DNA IQ™ spin basket (Cat.# V1221) was placed into the tube. The lysis buffer and filter card sample were transferred to the spin basket and centrifuged at room temperature for 2 minutes at maximum speed in a microcentrifuge. The spin basket was carefully removed and sample was transferred into well 1 of the Maxwell® 16 instrument for automated DNA extraction. Elution tubes (0.5ml) were placed into the elution tube holder at the front of each cartridge and 25–50µl of elution buffer was added to each. The program was started after installing the LEV Hardware into the Maxwell® 16 Instrument and choosing ‘Forensic’ mode. The finished samples were stored at 4°C if they were to be used shortly after, or at -20°C if they were to be stored for a longer time.

2.3.1.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a method of reproduction and detection of nucleic acids developed in 1987 by Mullis [64]. The availability of purified DNA polymerases and chemically synthesized DNA oligonucleotides has made it possible to exponentially amplify specific DNA sequences rapidly in vitro without the need for a living cell [65]. The technique allows the DNA from a selected region of a genome to be amplified a billion-fold, provided that at least part of its nucleotide sequence is already known. First, the known part of the sequence is used to design two synthetic DNA oligonucleotides, one complementary to each strand of the DNA double helix and lying on opposite sides of the region to be amplified. These oligonucleotides serve as primers for in vitro DNA synthesis, which is catalyzed by a thermostable DNA polymerase. This

was derived from the bacterium Thermus aquaticus. The molecular weight of the polymerase was 94 000 Da, the optimum pH was 7-8, the optimum temperature was 75-80° C. The half-life was 130 min at 92.5° C. The enzyme had a polymerization rate of 150 nucleotides per second. This Taq polymerase error rate was one of 180 bases to 10,000 base pairs. The entire PCR reaction was carried out in three stages; first the denaturation, then the hybridization or annealing of the primer and finally the extension. The hybridization temperature was varied according to the primers used [65].

PCR Mix	
MangoTaq (5 u/µl)	0.2 µl
5x PCR Mango Buffer	10 µl
$MgCl_2$ (50mM)	1.5 µl
dNTPs (10 mM)	0.25 µl
Forward primer (50 µM)	0.25 µl
Reverse primer (50 µM)	0.25 µl
Betain 5 M	10 µl
DNA template	5 µl
H2O	22.55 µl
Total volume	50 µl

In the PCR the double stranded DNA was first denatured (95 °C, 2 minutes).
This was followed by 40 cycles with 3 steps each:

- Denaturation (95 ° C, 30 seconds)
- Annealing of the primer (Temp. differs according to primer, 30 seconds)
- Elongation (72 °C, 1 minute)

Finally followed by a final elongation (72 °C, 8 minutes)

2.3.1.3 Gel electrophoresis

For separation of the DNA and control of PCR products, gel electrophoresis was performed. The principle of gel electrophoresis is that nucleic acid molecules are separated by applying an electric force to move the negatively charged molecules through the agarose gel. This is counteracted by a frictional force, which is dependent

on the size of the molecule and the viscosity of the solution. Because DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance travelled is inversely proportional to the log of its molecular weight [66]. One gram agarose was dissolved in 100 ml TBE buffer (45 mM Tris-borate, 1 mM EDTA) and boiled in the microwave. The concentration of the agarose thus depended on the size of the expected product. The product size being always less than 1000 bp, 1% gel was used. After the solution has cooled to about 60 °C, 2.5 µl ethidium bromide was added and mixed by circular movement of the flask. The agarose mix is filled into a horizontal gel chamber with a comb to a level of 0.5 – 1 cm. The gel was allowed to set and polymerize at room temperature and then the comb was removed, leaving out pockets. The gel was wrapped in plastic wrap and stored at 4 °C until used for maximum storage duration of 2 weeks. The electrophoresis was carried out using Horizontal Electrophoresis System (Whatman, Biometra, Goettingen) at 100 volts. The sample moved from the cathode to the anode. Gel documentation was done using Gene Flash Gel documentation system (Syngene Bio Imaging) and acquired images were printed using Video graphic printer UP-897 MD (Sony).

2.3.1.4 Exo/SAP purification

Exo/SAP purification is a method used to purify PCR product from unwanted remaining dNTPs and primers in order to optimize it for sequence reaction. It utilizes 2 hydrolytic enzymes namely exonuclease I (Exo) and shrimp alkaline phosphatase (SAP) and hence the name Exo/SAP.

EXO/SAP Purification	
PCR Product	8 µl
Enzyme Mix	
• dH2O	2,5 µl
• SAP	0,32 µl
• Exo I	0,18 µl
Total volume	11 µl

Cycler used was GeneAmp PCR System 9700 (Perkin Elmer). First step in cycler program was activation (37°C, 35 minutes) then inactivation (80°C, 15 minutes) then hold at 4°C.

2.3.1.5 DNA sequencing

This is a dideoxy chain termination method of Sanger using fluorescence-labeled dideoxynucleotides [67]. The principle of this reaction is the interruption of synthesis of the complementary strand by the incorporation of dideoxynucleotides (ddNTP). An extension of the DNA by the DNA polymerase is no longer possible, since the OH group is missing at the 3' carbon atom of the deoxyribose at the link with the phosphate group of the next nucleotide. It develops DNA fragments of different lengths.

DNA Sequencing	
5x Sequencing Buffer	1.5 µl
BigDye® Terminator v3.1 Ready Reaction	1 µl
Primer (5 µM)	1 µl
DNA Template (Exo/SAP product i.e. purified PCR)	3 µl
dH2O	3.5 µl
Total volume	10 µl

The sequencing reaction was carried out with GeneAmp PCR System 9700 cycler (Perkin Elmer) as a 30-cycle reaction:

- Denaturation: 95°C, 30 s (initial 1 min)
- Annealing: 55 °C, 15 s
- Elongation: 60 °C, 4 min (final 7 min)

The product was mixed with 1/5 volume of 1.5 M sodium acetate precipitated (pH 5.5) and twice the volume of 96% ethanol and centrifuged (30 min, 10,500 rev/min, 15°C). The pellet was washed with 200 µl of 70% ethanol and centrifuged (15 min, 10,500 rev/min, 15°C), and left to dry for 30 min at 60 °C.

The sequences were created using the Genetic Analyzer 3130xl (SOP QS1, 7 V1). The samples were stored at -20 ° C till they were loaded into the sequencer.

3 Results

3.1 SF-1 mutations in patients

NR5A1 analysis revealed 3 novel heterozygous mutations of the coding region in 3 out of 50 patients: c.184 C>T (p.Arg62Cys), c.361delGAGACAGG (p.Glu121AlafsX25) and c.460 G>A (p.Ala154Thr) (*Figure 9*). A review of the literature on http://www.pubmed.com and http://www.ncbi.nlm.nih.gov using search words (SF-1, NR5A1, DSD, Hypospadias, 46,XY) showed that the mutations were not previously published. Nor were they found on single nucleotide polymorphisms (SNPs) databases including Ensembl (http://www.ensembl.org/Homo_sapiens/Info/Index), db SNP (http://www.ncbi.nlm.nih.gov/SNP) or Exome Variant Server (http://evs.gs.washington.edu/EVS).

We used the following programs to detect potential splice site positions of the gene: Human Splicing Finder Version 2.4.1 (http://www.umd.be/HSF) [68], Fruitfly - Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html) [69] and SplicePort (http://spliceport.cbcb.umd.edu) [70]. Mutational analysis and prediction programs used include: Human Splicing Finder Version 2.4.1 (http://www.umd.be/HSF) [68], UCSC Genome Bioinformatics (http://genome.ucsc.edu/) [71, 72] and Mutation Taster (http://www.mutationtaster.org) [73].

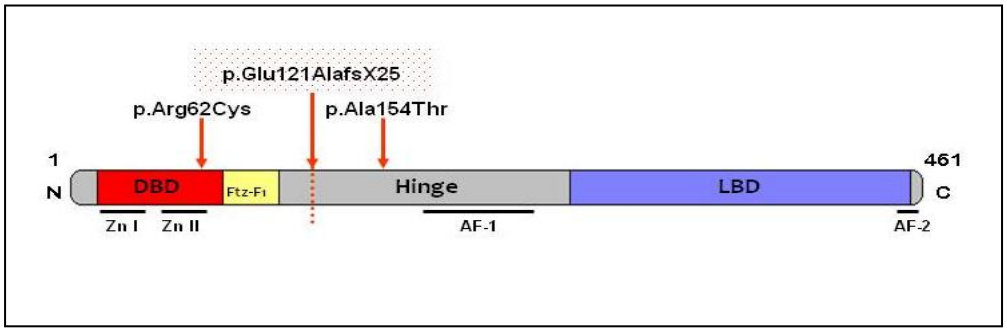

Figure 9: Cartoon of the structure of SF1 with the 3 mutations detected in this study.

3.1.1 Patient 1: Mutation c.184 C>T (p.Arg62Cys)

A 1 6/12 years old male**,** born to non-consanguineous parents (*Figure 10*) presented with severely hypoplastic phallus (stretched penile length was 1cm, SDS <-2.5), penile hypospadias, hypoplastic scrotum and impalpable gonads. Pregnancy and delivery

history were unremarkable. Pelvi-abdominal ultrasonography revealed two small testes, each 8-9mm in length, at medial ends of respective inguinal canals and no Müllerian structures. The patient was operated upon at 6 months, 12 months and 16 months of age for orchidopexy, correction of hypospadias, circumcision and plastic surgery. Basal testosterone (T) was low normal for age, while T peak after hCG stimulation (5000 IU/m^2 divided over 3 days) was low. LHRH stimulation test showed slightly elevated FSH post stimulation. Anti-Müllerian hormone (AMH) and inhibin B were both diminished (*Table 4*). Patient had normal cortisol and dihydroepiandrostenedione (DHEA-S) levels, and showed no signs or symptoms of adrenal affection.

Mutational analysis revealed a heterozygous c.184 C>T (p.Arg62Cys) mutation located in exon 3 in the DNA-binding zinc finger II region (*Figure 11A*). The exchange of cytosine with a thymine in this position causes a change of the amino acid sequence and conformational changes of the SF-1 protein which alters its features and hence is predicted to be disease causing. The arginine in position 62 is highly conserved in all species studied to date (*Figure 11B*). It is extremely important for proper binding of the protein to the DNA. It has H-bonds with the DNA and stabilizes the neighboring Zn-finger also via H-bonds and an aspartate (*Figure 12*). One hundred Egyptian controls (200 alleles) did not harbour this mutation. The mode of inheritance of this mutation remains unknown as the parents' DNA was not available for sequencing. Functional analysis of this mutation showed an aberrant transactivation of the known SF1-responsive reporters (human *AMH* promoter, human CYP11A1 promoter and mouse Tesco core enhancer of *Sox9*) in human embryonic kidney HEK293-T cells. The p.Arg62Cys mutant protein shows a significant reduction in its ability to stimulate either reporter gene activity (data not shown, functional analysis efforts attributed to other researcher) [74].

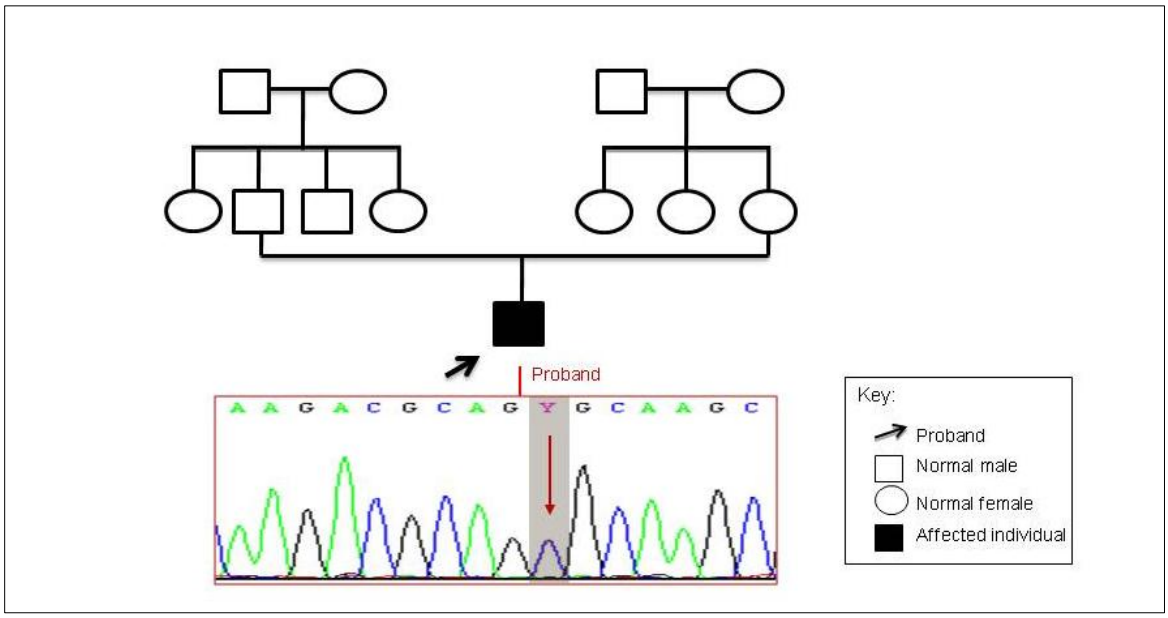

Figure 10: Family pedigree of patient 1 with chromatograph showing the heterozygous C to T transversion in proband.

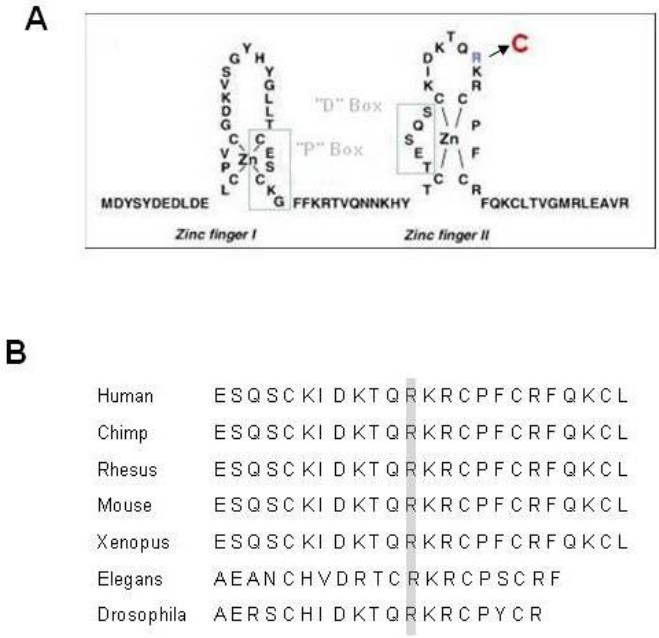

Figure 11: The p.Arg62Cys mutation.

(A) Part of the amino acid sequences of zinc fingers I and II showing the transversion of arginine to cysteine. Faded boxes indicate the P and D boxes. **(B)** The mutated arginine at position 62 is highly conserved in homologues of SF1 from all species studied to date.

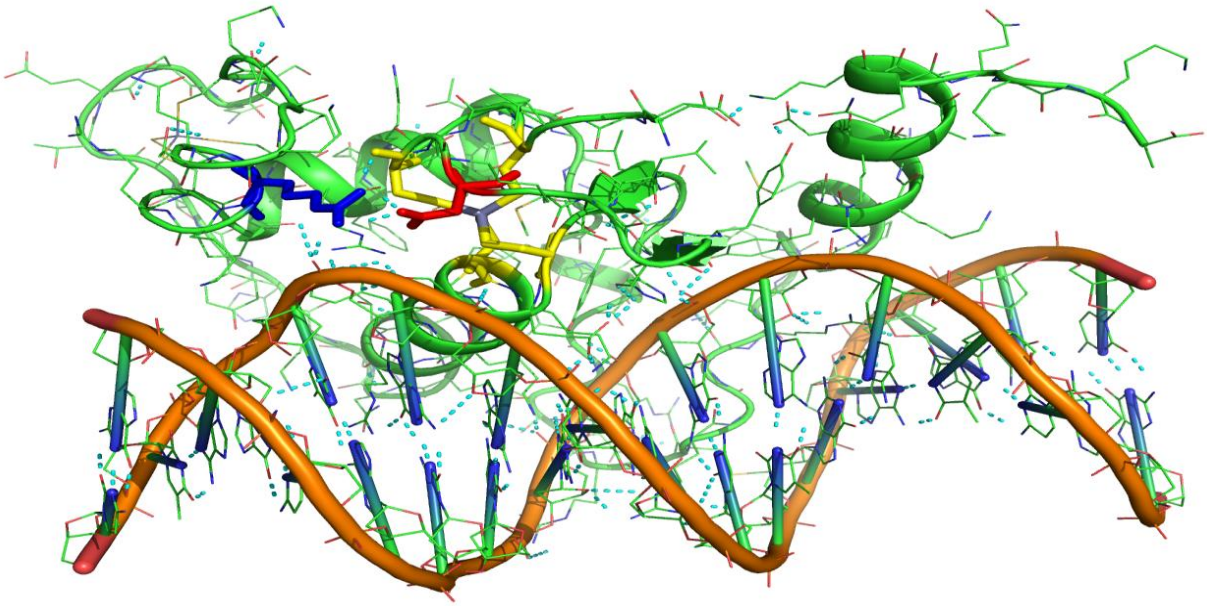

Figure 12: 3D structure of DBD (green) of SF-1 including the arginine at position 62.

The Arg62 has H-bonds (blue) to the DNA and stabilizes the neighboring Zn-finger (yellow) via H-bonds and an aspartate (red).

3.1.2 Patient 2: Mutation c.361delGAGACAGG (p.Glu121AlafsX25)

A 13-year-old male**,** born to non-consanguineous parents (*Figure 13*), presented with severely hypoplastic phallus (stretched penile length was 3.5cm, SDS <-2.5) and impalpable right testis. Left testis was descended and of average size. Pregnancy and delivery history were unremarkable. Pelvic laparoscopy revealed atrophic right testis and no Müllerian structures. He was operated upon for correction of hypospadias and bifid scrotum. T peak post HCG stimulation was low for age. AMH and inhibin B were very low for age and Tanner stage (*Table 4*). Penile length increased to 5.5 cm on testosterone enanthate injections. Patient showed no signs or symptoms of adrenal affection.

Mutational analysis revealed a heterozygous 8bp deletion mutation c.361delGAGACAGG (p.Glu121AlafsX25) causing frameshift resulting in a stop codon in exon 4 predicted to produce either a severely truncated protein or no protein at all through nonsense-mediated mRNA decay. One hundred Egyptian controls (200 alleles) did not harbour this mutation. This appears to be a de novo mutation, as neither parent harbours it.

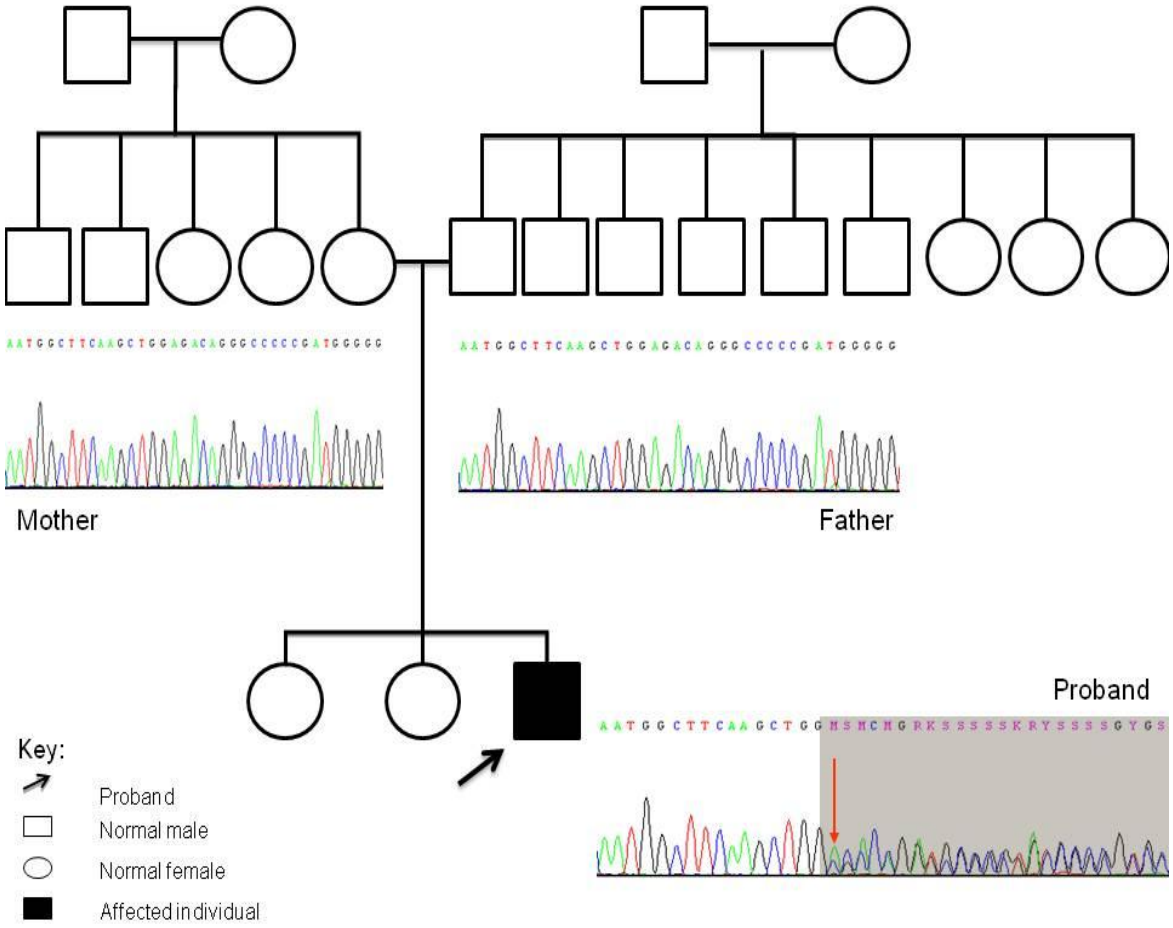

Figure 13: Family pedigree of patient 2 with chromatographs showing the p.Glu121AlafsX25 mutation in the proband and wild type in the parents.

3.1.3 Patient 3: Mutation c.460 G>A (p.Ala154Thr)

A 4-year-old male, born to consanguineous parents (first cousins once removed) (*Figure 14*), presented with severely hypoplastic anchored phallus (stretched penile length was 2.5cm, SDS <-2.5) and penile hypospadias. Both testes were of normal size in scrotum. Pregnancy and delivery history were normal. Hypospadias was surgically corrected. Basal T was normal for age, while peak T post stimulation with hCG was low. FSH post stimulation, Inhibin B and AMH levels were normal (*Table 4*). Patient showed no signs or symptoms of adrenal affection.

Mutational analysis revealed a heterozygous c.460 G>A (p.Ala154Thr) mutation at the hinge region in exon 4. The hinge region is important for transcriptional capacity of SF-

1, as it contains a phosphorylation site and a synergy control motif with sumoylation sites and hence it is predicted to reduce the transcriptional capacity of the protein. The amino acid, as well as nucleotide in this position, is conserved in human, chimp and rhesus but not in other species (*Figure 15*). Functional analysis of this mutation showed no reduced transactivation of the known SF1-responsive reporters (human *AMH* promoter, human CYP11A1 promoter and mouse Tesco core enhancer of *Sox9*) in human embryonic kidney HEK293-T cells (data not shown, functional analysis efforts attributed to other researcher) [74], therefore we did not consider the mutation to be alone relevant for the phenotype and did not include it in the percentage of mutations detected in our study. The father harbours the same mutation in the heterozygous form. He has fathered two more healthy girls and clinical exam showed normal male genitalia with a penile length of 10 cm, both testes size 25 and Tanner stage P5 A3. The mother's sequence was wild type. Although, patient 3's parents were first cousins once removed, he did not harbour a homozygous mutation (*Figure 14*). The consanguinity rate among parents of all 46,XY DSD patients included in our study was 53%, where 34% were first cousins and 19% were second cousins or further relatives.

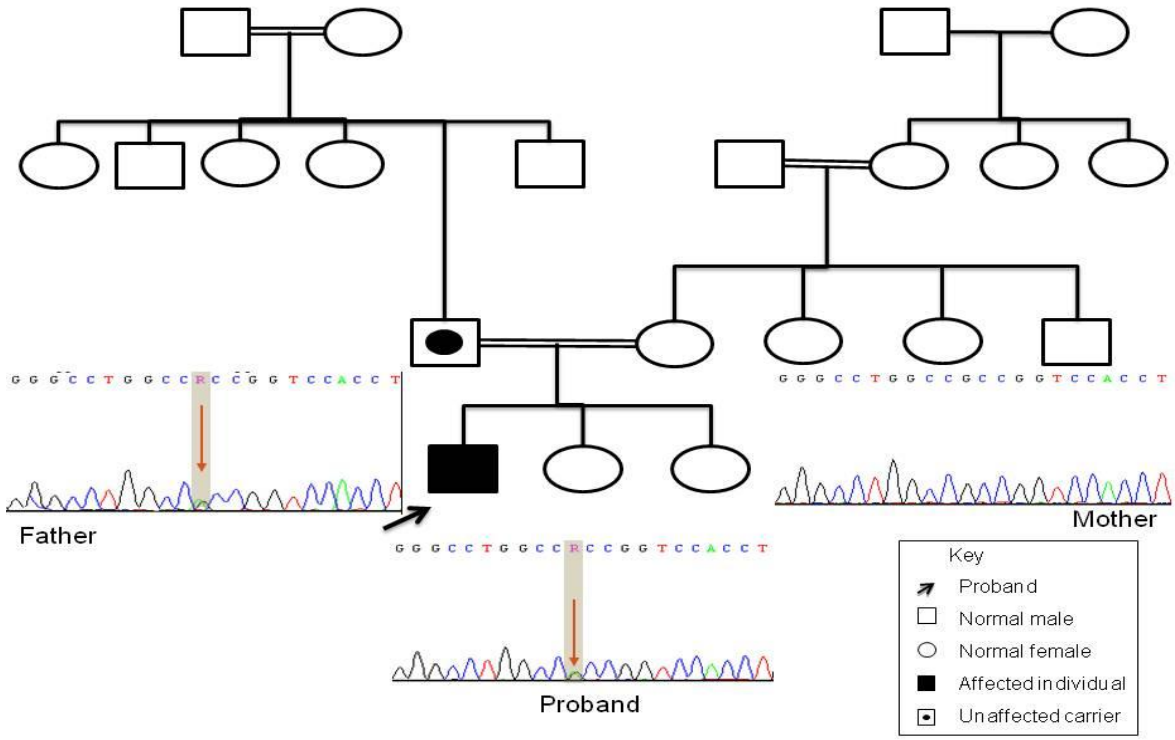

Figure 14: Family pedigree of patient 3 with chromatographs showing the p.Ala154Thr mutation in the proband and the father, and wild type in the mother.

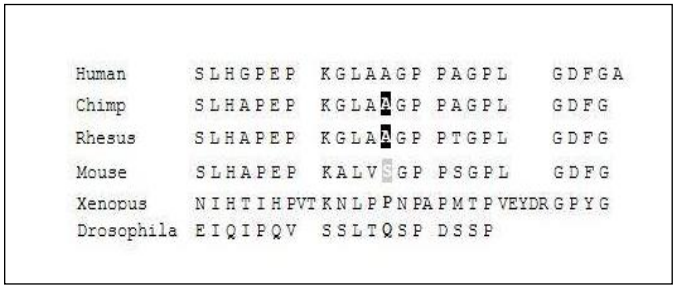

Figure 15: The p.Ala154Thr mutation conservation.

The mutated alanine at position 154 is not conserved in homologues of SF-1 from Mouse, Xenopus and Drosiphila species.

Table 4: Clinical, hormonal and molecular data of patients with *NR5A1* mutations

Patient	Age in years	External genitalia	Gonads	Uterus	Epididymis, vas deferens	Hormones	Gender Assignment	Adrenal affection/ investigations	Mutations (heterozygous)	Parents
1	1 6/12	Penile hypospadias, hypoplastic phallus (1cm, SDS <-2.5), hypoplastic scrotum	Bilateral small inguinal testes (each 8-9mm length)	No	Yes	At 4 months: T 0.1→ 1.8 ng/mL(↓) FSH 1.3 → 9.1 mIU/mL(↑) LH 0.5 → 2.2 mIU/mL At 3 years: Inhibin B 15.5 ng/L (↓) AMH 10.6 ng/mL (↓)	m	Cortisol 9,9 µg/dL (N) DHEAS 12,4 µg/dL (N)	p.Arg62Cys (Exon 3) + p.Gly146Ala (Exon 4)	N/A
2	13	Hypospadias, hypoplastic phallus (3,5cm, SDS <-2.5), bifid scrotum	Right atrophic undescended testis. Left testis normal in scrotum	No	Yes	At 13 years: T post hCG 0.75 (↓) Inhibin B 9.0 (↓) AMH <0.10 (↓)	m	NA, No symptoms of adrenal affection	p.Glu121AlafsX25 (Exon 4)	Mother WT Father WT
3	4	Penile hypospadias, hypoplastic phallus (2,5cm, SDS <-2.5), anchored	Both testes normal in scrotum	No	Yes	At 4 years: T 0.18 →1.6 (↓) FSH 1.2 →8.7 (N) LH 0.6 →2.1 (N) Inhibin B 70.4 (N) AMH >20 (N)	m	NA, No symptoms of adrenal affection	p.Ala154Thr (Exon 4) (no reduced function)	Mother WT Father p.Ala154Thr

Conversion to SI units: Testosterone [ng/mL] x 3.47 = [nmol/L]; FSH and LH [mIU/mL] x 1 = [IU/L]; AMH [ng/mL] x 7.14 = [pmol/L]; DHEAS [µg/dL] x 0.027= [µmol/L]. Testosterone levels are basal and post hCG stimulation. FSH and LH levels are basal and 90 minutes post LHRH stimulation.
Normal values:
Basal testosterone in males with; Tanner stage 1 <7 years: <0,11-0,65; Tanner stage 2-3: 0.62-2.26 ng/ml; Tanner stage 4-5: 1.68–6.0 ng/ml;
Normal testosterone peak after stimulation with HCG 5000 IU/m^2 at 72 hours 3.0-10.0 ng/ml;
Basal FSH in males Tanner stage 1: <1–1.3 IU/l, Tanner stage 2-3: <1–4.0 IU/l, Tanner stage 4-5: 1.4–5.1 IU/l;
Basal LH in males Tanner stage 1: <1–1.5 IU/l, Tanner stage 2-3: 1–4.1 IU/l, Tanner stage 4-5: 3.4–7.5 IU/l;
Inhibin B reference values: see reference [75]
AMH [76]: In childhood median = 105.7 ng/mL, range (55.3-186.9 ng/mL); Prepubertal < 9 years = 97,58 (44,94-170,52); Prepubertal > 9 years = 81,9 (41,58-155,82); Tanner G2 = 35,42 (6,44-156,8); Tanner G3 = 9,24 (3,08-102,76); Tanner G4 = 6,02 (2,1-15,68); Tanner G5 = 6,86 (3,22 - 17,92).
NA: Not available. (↑): high. (↓): low. (N): normal

3.2 Single nucleotide polymorphisms (SNPs) and novel intronic variants

3.2.1 Novel intronic variants

Two novel heterozygous variants were detected in intron 6 in 2 patients (Patients 4 and 5) (*Figure 16*).

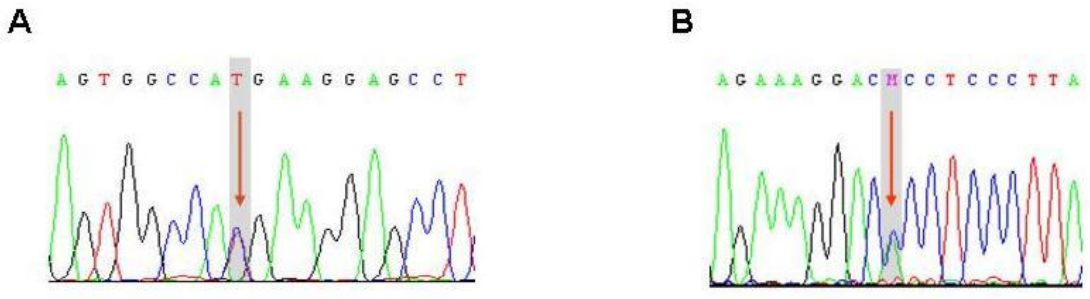

Figure 16: Two novel intronic variants.
(A) Chromatograph showing the heterozygous C to T transversion in intron 6 causing the intronic variant c.1137+105 C>T in Patient 4. **(B)** Chromatograph showing the heterozygous C to A transversion in intron 6 causing the intronic variant c.1137+129 C>A in Patient 5.

3.2.1.1 Intronic variant c.1137+105 C>T

This heterozygous cytosine/thymine exchange was detected 105 base pairs behind exon 6 in Patient 4 (*Figure 16A*). It was not previously described in literature or in SNPs databases. It was also not found in 100 Egyptian controls. Patient 4 inherited this variant from his healthy father, as he harboured the same variant in the heterozygous form, while the mother was wild type for this gene. Analysis of this variant using different splicing prediction programs confirmed that it does not lie in any of the splicing sites of this intron and hence does not affect protein splicing. Mammalian base-wise conservation score and GERP score for mammalian alignment were both very low, as this base position was not conserved in different species (*Figures 17 and 18*). Since this variant is not conserved, and does not lie in any of the splicing sites of this intron, we considered it non-pathogenic.

3.2.1.2 Intronic variant c.1137+129 C>A

The heterozygous cytosine/adenine exchange was detected 129 base-pairs behind

exon 6 in patient 5 (*Figure 16B*). This was not documented in the SNP databases or in literature. Analysis of this variant using different splicing prediction programs confirmed that it does not lie in any of the splicing sites of this intron and hence does not affect protein splicing. This position is conserved in human, rhesus, mouse, dog, and elephant but not in chicken species, conservation scores were moderately high (*Figures 17 and 18*), however we could detect it in 1 out of 100 healthy Egyptian control, so we could not consider it as pathogenic.

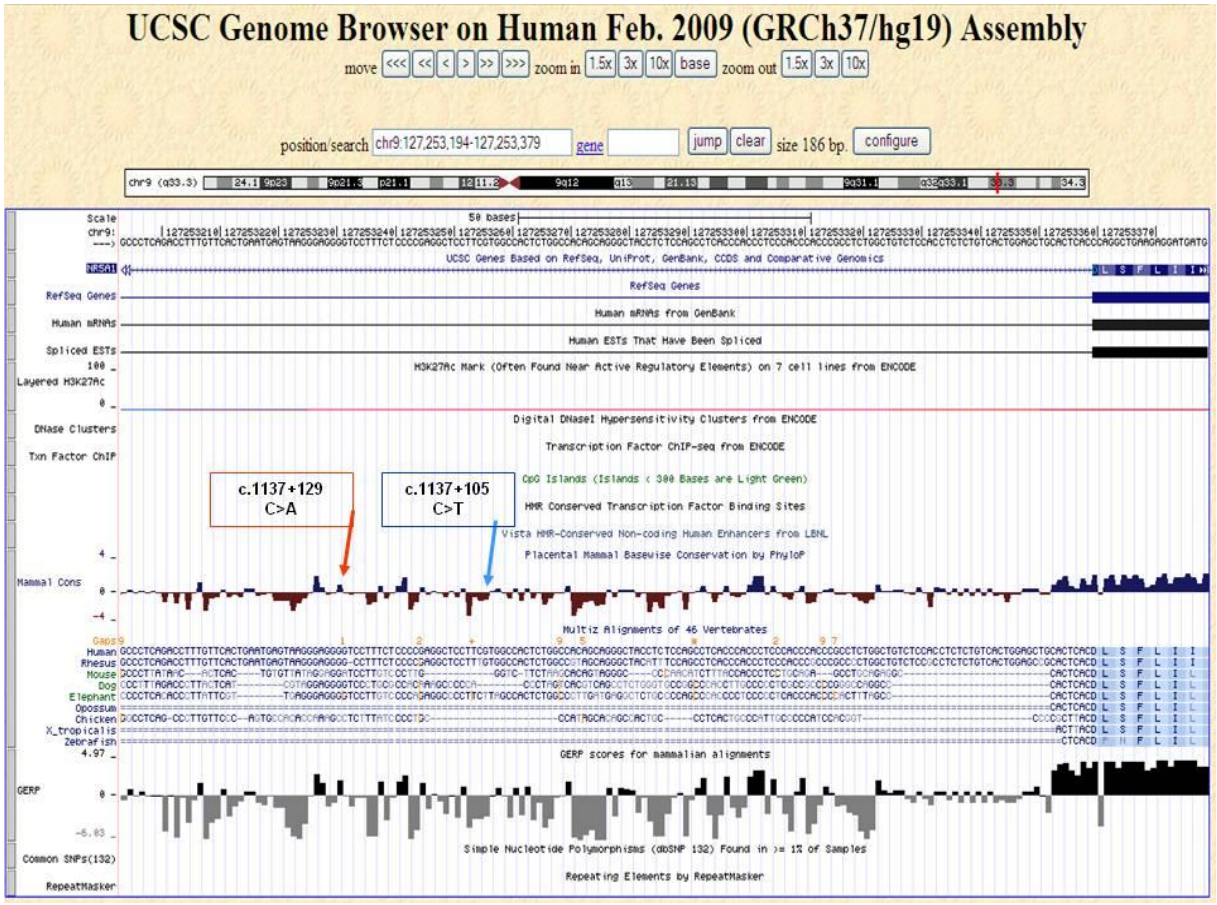

Figure 17: Overview of conservation of 2 intronic variants.

Note that the *NR5A1* is a reverse strand. Picture adapted from UCSC Genome Bioinformatics website (http://genome.ucsc.edu/) [71, 72]

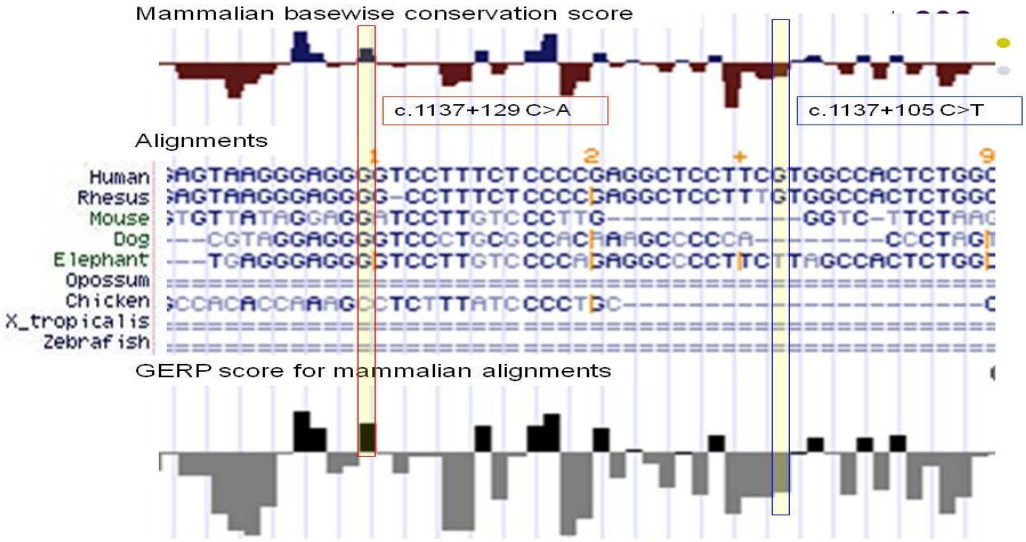

Figure 18: Details of conservation of 2 intronic variants.

Note that the *NR5A1* is a reverse strand. Picture adapted from UCSC Genome Bioinformatics website (http://genome.ucsc.edu/) [71, 72]

3.2.2 Previously described SNPs

Six different single nucleotide polymorphisms were detected in our patients (*Table 5*). They were found in both heterozygous and homozygous forms. The p.Gly146Ala (c.437G>C, rs1110061) polymorphism was found in 17 patients (34%) in heterozygous state and in 31 controls among which 5 were in homozygous state. Other single nucleotide polymorphisms detected in our study include p.Pro125Pro, p.Leu451Leu, c.871-20C>T, c.*82C>T and IVS4-13. The p.Pro125Pro (c.375G>A, rs1110062) in exon 4 was found in 4 patients (3 heterozygous, 1 homozygous) and 9 controls (8 heterozygous, 1 homozygous). The c.871-20C>T (rs2297605) in intron 4 was detected in 27 patients (21 heterozygous, 6 homozygous) and in 65 controls (50 heterozygous, 15 homozygous). The c.*82C>T (rs915034) in intron 7 was detected in 32 patients (18 heterozygous, 14 homozygous) and was not sequenced in controls. The IVS4-13 (c.871-13C>T, rs189724865) in intron 4 was found in 1 patient in heterozygous form and in none of the controls.

Table 5: Single nucleotide polymorphisms detected in the study

	p.Gly146Ala	p.Pro125Pro	IVS4-13	c.871-20bp C>T	p.Leu451Leu	c.*82 C>T
Exon/Intron	exon 4	exon 4	intron 4	exon 5	exon 7	intron 7
cDNA position	c.437G>C	c.375G>A	c.871-13C>T	c.871-20bp C>T	c.1353G>A	c.*82 C>T
Genomic DNA position	g.127262802 C>G	g.127262864 C>T	g.127255441 G>A	g.127255448 G>A	g.127245070 C>T	g.127244955 G>A
RefSNP (RS)	1110061	1110062	189724865	2297605	79833327	915034
Minor allele frequency (MAF)	0.272	0.076	0.0014	0.424	0.010	0.370
Percentage detected in controls	31%	9%	0%	65%	NA	NA
Percentage detected in patients	34%	8%	2%	54%	2%	64%

4 Discussion

4.1 Clinical characteristics of the patients with mutations in this study

Here, we present three novel heterozygous SF-1 mutations in a cohort of 50 Egyptian 46,XY DSD patients with mild to severe underandrogenization. All three patients with *NR5A1* mutations were presenting with penile hypospadias and hypoplastic phallus. Patients 1 and 2 (p.Arg62Cys and p.Glu121AlafsX25) also displayed maldescended testes and partial gonadal dysgenesis with impaired Leydig cell and Sertoli cell functions (low testosterone, low inhibin B and AMH). Patient 3 (p.Ala154Thr) showed normally descended testes, only mildly impaired Leydig cell and normal Sertoli cell function (slightly decreased testosterone, normal inhibin B and AMH) (*Table 4, Figure 19*).

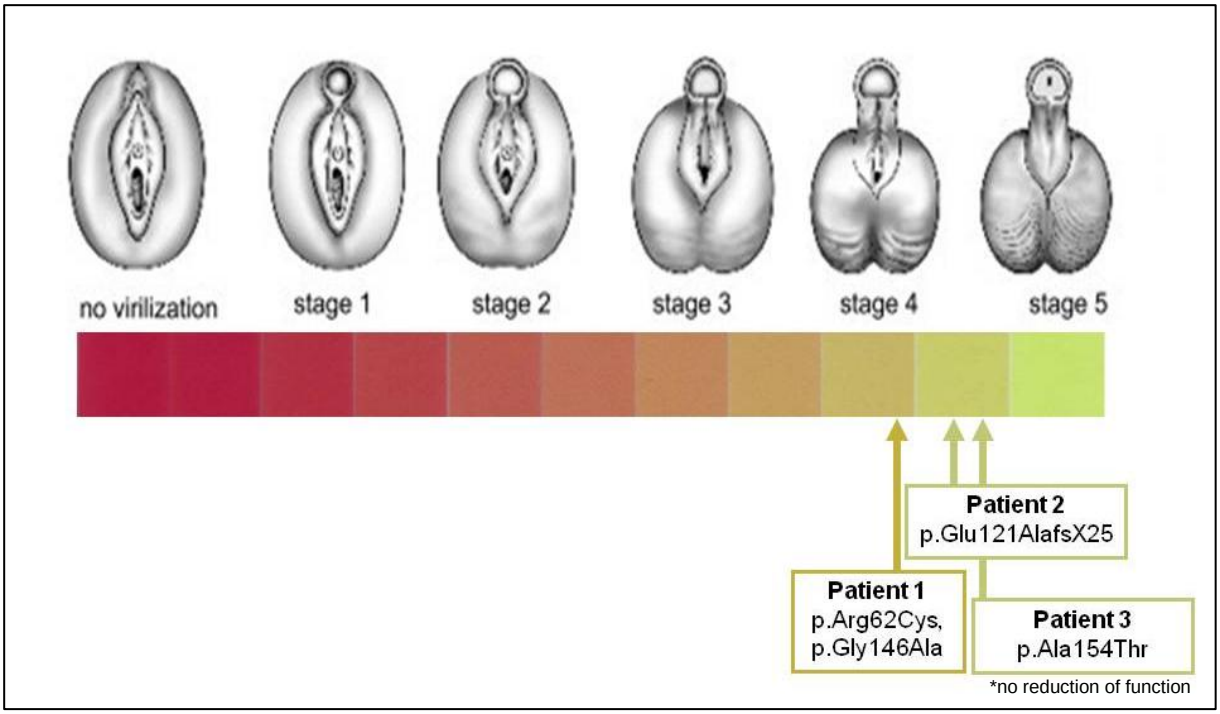

Figure 19: Illustration of phenotypes of patients with SF-1 mutations detected in this study according to Prader staging.

With SF-1 mutations, often there is biochemical evidence of partial gonadal dysgenesis and significantly impaired androgen synthesis as shown by low levels of testosterone, inhibin B and AMH, and an elevation of FSH [77]. Our patients demonstrated moderate underandrogenization at birth showing that Leydig cell function was already disturbed

during prenatal life. In contrast, embryonic Sertoli cell function was sufficient for adequate AMH production and subsequent Müllerian duct regression prenatally, as none of the patient displayed a uterus or uterine remnant. In patients 1 and 2 however, AMH and inhibin B levels were diminished/undetectable at 3 and 13 years, respectively, and FSH was elevated, revealing Sertoli cell hypofunction. This could be the result of disease progression, as previously described in 46,XY patients with SF-1 mutation and progressive deterioration of the Sertoli cell function with age [52, 78]. Moreover, the small testes at diagnosis in patient 1 and unilateral testicular atrophy in patient 2 reflect gonadal dysgenesis.

Patient 3 seems to be mildly affected as demonstrated by his phenotype and his hormones. He showed a mild underandrogenized phenotype of hypoplastic phallus and penile hypospadias with normal descended testes. He had a slightly diminished testosterone response to HCG stimulation at 4 years of age, with normal AMH and Inhibin B levels and FSH post LHRH stimulation (*Table 4*). The mild phenotype of the patient cannot be explained by the mutation, as it did not cause reduced transcriptional activity of the protein in-vitro in a later study [74]. However, normal testosterone production in 46,XY DSD patients should not exclude SF-1 as a cause of the disease especially given that it has been recently reported that patients with SF-1 mutations can have normal/high testosterone production [52, 78-80]

4.2 Phenotypes of SF-1 mutations

4.2.1 SF-1 mutations with adrenal disorders

Early studies on the potential role of SF-1 in humans were based on the *Nr5a1* knock-out mouse studies (see Introduction p. 6-7) which highlighted the phenotype of XY complete gonadal dysgenesis and primary adrenal failure [77]. To date, only 2 cases were described with this phenotype, where disruption of key DNA-binding motifs of SF-1 seemed to have occurred [81]. The first human described with this phenotype showed a *de novo* heterozygous p.G35E mutation in SF-1. This child was a 46,XY phenotypically female who presented in the first 2 weeks of life with a severe salt-losing crisis and adrenal failure (cortisol 1.2 µg/dl) [38].

The second and last case of SF-1 mutation in 46XY with adrenal failure was reported in 2002 [39]. A homozygous p.R92Q was detected in a phenotypically female baby who presented shortly after birth with a hypoglycaemic convulsion and evidence of progressive primary adrenal failure in the neonatal period (hyponatremia, hyperkalemia, marked hyperpigmentation, hypotonia, weight loss, and failure to thrive). Abdominal CT scan demonstrated left adrenal hypoplasia and right adrenal agenesis [39]. Al-Khairi and Achermann explained that this report provided credible proof of a functional gene dosage effect of SF-1 influencing both adrenal and gonadal development and function: The fact that the inheritance was recessive and that the mutation was present in a homozygous state in the affected child meant that other molecular mechanisms such as dominant negative effects, mosaicism, skewed allelic expression, or even a covert second mutation were not implicated. The authors also pointed out the absence of complete loss of function of SF-1 in humans to date [81]. Mallet et al. discussed SF-1 haploinsufficiency and suggested that: SF-1 acts in a dose-dependent manner, while a 50% dose of normal protein is sufficient to permit adrenal development and function, a residual dose of less than 50% is not sufficient for the development of both adrenal and gonad. They suggested that human adrenal development is more resistant to the effects of SF-1 haploinsufficiency than testis development [42]. This could explain why most humans with *NR5A1* mutations have altered gonadal function with apparently normal adrenal function [82]. Additionally, some studies have demonstrated a dominant negative effect of SF-1 mutations in humans [83, 84]. Therefore, it has been proposed that heterozygous mutation impaired adrenal development only if the two mechanisms, gene dosage and dominant negative effects, occur [42].

In 2000, Biason-Lauber and Schoenle reported a *de novo* heterozygous *NR5A1* mutation in a 46,XX phenotypically normal girl who presented at age 14 months with adrenal insufficiency and seizures after having had otitis and tonsillitis one week earlier. The diagnosis of adrenocortical insufficiency was based on the clinical picture of severe slackness, muscular hypotonia, hyponatremia, hyperkalemia, elevated ACTH and inadequately low cortisol [40].

Excluding the 3 infants reported with SF-1 mutations and adrenal disorders [38-40], all studies on SF-1 mutations in humans reported no adrenal affection. None of the three

patients in this study showed any signs or symptoms of adrenal affection to date excluding severe adrenal insufficiency.

4.2.2 The 'typical' 46,XY DSD phenotype

An increasing number of heterozygous changes in *NR5A1* have been identified in patients with 46,XY DSD displaying the 'typical' phenotype of ambiguous genitalia (or clitoris hypertrophy) at birth, a urogenital sinus, small inguinal testes, absent or rudimentary Müllerian structures and intact or rudimentary Wolffian structures without adrenal insufficiency [46, 47, 49, 85-89] *(Table 6)*. Often hormonal evaluation showed evidence of partial gonadal dysgenesis demonstrated by low levels of inhibin B, AMH and testosterone and an elevation of FSH [77].

One of our patients (Patient 1, p.Arg62Cys) displayed the 'typical' SF-1 phenotype. He displayed a 1 cm long phallus (at 1 6/12 years, <-2.5 SDS) with penile hypospadias and hypoplastic scrotum, bilateral small inguinal testes and no Müllerian structures. He also displayed evidence of partial gonadal dysgenesis (*Table 4*). Patient two, who harboured the p.Glu121AlafsX25 frameshift mutation, displayed a less severe phenotype with hypoplastic phallus (3.5 cm long at 13 years of age, <-2.5 SDS), hypospadias, bifid scrotum, unilateral cryptorchidism and no Müllerian structures and partial gonadal dysgenesis. Patient 3 with the p.Ala154Thr mutation, causing no reduction of protein function, showed the mildest phenotype among our three patients. He displayed a hypoplastic phallus (2.5 cm at 4 years of age, <-2.5 SDS) with penile hypospadias and normal descended testes (*Figure 19*). Gonadal dysgenesis could not be found, as hormonal evaluation showed low testosterone, but normal Inhibin B, AMH and FSH. Therefore, it can be hypothesized that changes in SF-1 are likely to predispose an individual to a DSD phenotype but the ultimate clinical picture most likely is influenced by other factors, as evidenced by the wide range of phenotypes associated with SF-1 mutations in 46,XY patients.

4.2.3 SF-1 mutations and primary amenorrhea

NR5A1 mutations have been detected in 46,XY females with complete/partial gonadal dysgenesis and no adrenal insufficiency *(Table 6).* Cases were mostly adolescents

presenting with primary amenorrhea and/or clitoromegaly [46, 48, 86, 87, 89]. In 2010, Philibert et al. reported *NR5A1* mutations in 5/15 46,XY female adolescents presenting with primary amenorrhea and low testosterone levels, 3 of which also had clitoromegaly, concluding that *NR5A1* mutations are a frequent cause of this severe phenotype [89]. No SF-1 mutations could be detected in this study in 13 patients with a similarly severe phenotype.

4.2.4 SF-1 mutations and hypospadias

NR5A1 mutations were reported in 46,XY patients with severe hypospadias and small undescended testes due to partial gonadal dysgenesis and/or defective androgen synthesis [44, 45, 47]. The first case was described by Lin et al. in 2007 and harbored a heterozygous p.L437Q mutation [47]. In 2009, Köhler et al. studied a cohort of 60 boys with hypospadias and detected 3 novel heterozygous *NR5A1* mutations [45]. All previously mentioned cases displayed the most severe form of hypospadias (penoscrotal) and undescended testes [45]. Only one patient has been reported with simple distal penile hypospadias [44]. Two (Patients 1 and 2) out of 23 hypospadias patients carried SF-1 mutations with reduced transcriptional function in our study, which represents a frequency of 8% of the hypospadias group. Patient 3 with the mild phenotype of penile hypospadias without signs of gonadal dysgenesis carried a mutation without decreased transcriptional activity of the protein. Therefore he was not included in the frequency calculated.

4.2.5 SF-1 and testicular atrophy

In 2007, a French collaborative study on a cohort of 24 boys with bilateral anorchia (vanishing testis syndrome) detected one patient with a heterozygous *NR5A1* (p.V355M) mutation. He presented with one absent and one very small testis with undetectable AMH in early infancy. Interestingly, his twin brother harbored the same mutation and displayed normal male external genitalia (Tanner of P5, G5 at 15 years) and had a normal puberty [50]. We did not detect any *NR5A1* mutations in 9 cases with bilateral atrophic testes and 1 case with unilateral atrophic testis.

4.2.6 SF-1 and male infertility

In the work of Bashamboo et al., *NR5A1* mutations were investigated in a cohort of 315 otherwise healthy men with severe spermatogenic failure. Seven heterozygous

missense hinge region mutations were detected. A quantitative reduction in the transactivation of both the Cyp11a1promoter and the AMH promoter was observed in transactivation assays of the mutations. These in vitro functional assays demonstrated that each mutation may lead to a functional disturbance of the SF-1 protein and may affect the regulation of its downstream target genes during gonadal development and function, which the authors suggested could have caused a mild form of gonadal dysgenesis in their patients as well as a progressive loss of gonadal function over time [52]. Another cohort of 488 infertile men was sequenced for *NR5A1* mutations by Röpke et al. in 2013 and revealed 3 novel heterozygous missense mutations in the hinge region of the SF-1 protein in 3 patients. The mutations led to substitutions of highly conserved amino acids and were predicted to be damaging to SF-1 protein function, however, no functional analysis of the mutations was done [53]. Most recently in 2015, Ferlin et al. reported *NR5A1* heterozygous missense mutations in 7/398 (1.8%) men with spermatogenic impairment, of which 4 were idiopathic and 3 with history of cryptorchidism. All 7 mutations detected were in exon 4, four of which are located in the hinge region and three in the ligand-binding domain of the protein [90].

4.2.7 SF-1 and ovarian insufficiency

Heterozygous *NR5A1* mutations have also been identified in 46,XX patients with primary ovarian insufficiency [46, 48] and premature ovarian failure [49]. These presented with either primary or secondary amenorrhea. Lourenco et al. reported a female with primary gonadal failure (elevated LH and FSH, low estradiol) whose ovarian biopsy showed severe fibrosis with absence of follicles [48]. Most of these women had been identified on account of a history of 46,XY DSD and POI in other family members. Through these patients a role of SF-1 factor in ovarian development and function could be demonstrated [77].

4.3 SF-1 genotype-phenotype correlation

A definite phenotype-genotype correlation of SF-1 mutations has not been described to date. The limitation is that identifying a pathogenic SF-1 mutation may not predict the clinical picture because the phenotype can be highly variable, even within the same

family [49, 91]. A family was previously reported with 6 members harbouring the same p.Y183X heterozygous mutation but displaying different phenotypes [49]. Four affected 46,XY siblings had 46,XY DSD or hypospadias as follows: one 46,XY DSD sibling displayed ambiguous genitalia and three displayed isolated hypospadias, two of which were severe perineal and one was mild glandular. An affected 46,XX sister had normal sexual development but increased FSH levels and the 46,XX mother had early menopause at 32 years possibly due to premature ovarian failure [49].

4.4 SF-1 hinge region mutations

The hinge region is important for post-transcriptional/translational modifications. In patients with severe forms of 46,XY DSD, previously described mutations are mostly missense mutations in the DNA-binding region (including its accessory DNA-binding domain) or in the ligand-binding domain as well as nonsense mutations leading to severe changes of the protein [38, 39, 44-47, 78, 85-88, 92].

So far, almost only nonsense mutations in the *NR5A1* hinge region have been reported in severe 46,XY DSD phenotypes [44, 46, 48, 49, 85, 86, 89], in hypospadias [45, 49], in 46,XX primary ovarian insufficiency [46, 48] and premature ovarian failure [49] (*Table 6*). In contrast, only missense mutations in the hinge region were detected in the minor DSD phenotype of male infertility [52, 53, 90]. It can be hypothesized that missense mutations of the hinge region result in a very mild phenotype. As the p.Ala154Thr mutant of the hinge region in this study did not show reduced transcriptional activity, in vitro interaction of the mutant with modifying factors affecting testosterone production through protein interaction might play a role in the development of hypospadias in this patient. Moreover, patient 3 inherited the p.Ala154Thr mutation from his unaffected father, who had a history of normal sexual activity and was able to father three children (*Figure 14*).

Phenotype Group*	SF-1 Mutations in hinge region	Author
Complete/severe gonadal dysgenesis female external genitalia with Müllerian structures	c.424_427dupCCCA/p.G146A	*Köhler et al., 2008 [86]*
	c.666delC	*Lourenco, 2009 [48]*
Ambiguous genitalia or virilised female ext genitalia with no (or NA) Müllerian structures	c.536delC	*Coutant et al., 2007 [85]*
	p.Y138X	*Köhler et al., 2008 [86]*
	c.390delG	*Lourenco, 2009 [48], Allali et al., 2011 [44]*
	p.P124PfsX24	*Philibert et al., 2010 [89]*
	p.Y183X	*Warman et al., 2011 [49]*
	p.L231_L233dup	*Camats et al., 2012 [46]*
	p.Q206TfsX20	*Camats et al., 2012 [46]*
	p.P130RfsX165	*Camats et al., 2012 [46]*
Hypospadias and cryptorchidism	p.Q107X	*Köhler et al., 2009 [45]*
Isolated hypospadias	p.Y183X	*Warman et al., 2011 [49]*
Male infertility	p.G123A + p.P129L	*Bashamboo et al., 2010 [52]*
	p.P131L	*Bashamboo et al., 2010 [52]*
	p.R191C	*Bashamboo et al., 2010 [52]*
	p.G212S	*Bashamboo et al., 2010 [52]*
	p.D238N	*Bashamboo et al., 2010 [52]*
	p.P210P	*Röpke et al., 2013 [53]*
	p.V240V	*Röpke et al., 2013 [53]*
	p.G165R	*Röpke et al., 2013 [53]*
	p.R114W	*Ferlin et al., 2015 [90]*
	p.V173M	*Ferlin et al., 2015 [90]*
	p.P210S	*Ferlin et al., 2015 [90]*
	p.Y211C	*Ferlin et al., 2015 [90]*
46,XX POI	c.666delC	*Lourenco, 2009 [48]*
	c.390delG	*Lourenco, 2009*
	p.L231_L233del	*Lourenco, 2009 [48]*
	p.G123A + p.P129L	*Lourenco, 2009 [48]*
	p.P235L	*Camats et al., 2012 [46]*
46,XX POF	p.Y183X	*Warman et al., 2011 [49]*
	* all patients had no adrenal failure	

Table 6: SF-1 mutations previously described in the hinge region and their phenotype

4.5 The p.Gly146Ala polymorphism

The *NR5A1* gene variation p.Gly146Ala (c.437G>C, rs1110061) lies in the hinge region of the *NR5A1* gene. Previous studies suggested that this polymorphism might be of clinical importance in increasing susceptibility to adrenal disease and to diseases of defective androgenization [93-95]. In 2003, WuQiang detected this variation in higher frequency in patients with adrenal disease (30%) than in controls (8.2%). Functional studies showed slightly diminished transactivation activity (~20% decreased activity) evidenced by both adrenal specific cyp11A promoter and ovary specific cyp19 promoter II. However, it did not affect protein expression or stability, displaying no dominant negative effect. It displayed normal interaction pattern with standard co-regulators and subnuclear distribution pattern, and was considered a non-synonymous single nucleotide polymorphism, since it occurred in normal controls as well [95]. In 2005, Wada et al. studied the polymorphism in 46,XY patients with micropenis. They detected heterozygous p.Gly146Ala variation in a significantly higher frequency in patients with severe micropenis (50%) than in controls (20.8%), however the frequency in the mild micropenis group was lower than in controls. The homozygous form was detected in patients as well as in controls; the lowest frequency was detected in the severe micropenis group [94]. In 2006, Wada et al published yet another report on p.Gly146Ala in patients with cryptorchidism, where they detected the heterozygous form in 47%, compared to 25.7% of controls; again the homozygous form was seen more frequently in controls than in patients [93].

In 2012, two different research groups reported the occurrence of the p.Gly146Ala variant significantly more often in 46, XX female patients with premature ovarian failure (POF) than in controls [96, 97]. Lakhal et al. found that in both POF patients and controls, a marked reduction in estradiol levels was obvious in the heterozygous and more so in the homozygous state, demonstrating dose-dependency in the contribution of Gly146Ala to the diminished estradiol levels [96]. Furthermore, it has been repeatedly reported as a SNP in cohorts of 46,XY DSD patients [43, 46, 86, 87, 90] and in 46,XX with POF [48] without functional analysis to contradict or confirm previous studies.

In the present study, the heterozygous p.Gly146Ala polymorphism was detected in 34% of 46,XY DSD patients (17/50 patients) compared to 26% of controls (26/100 controls),

while the homozygous form was detected in none of the patients and in 5% of controls. The results are similar to the two previous studies by Wada et al. where the heterozygous form occurred more often in patients and the homozygous form more often in controls [93, 94]. The international HapMap project (http://hapmap.ncbi.nlm.nih.gov) reported the heterozygosity of this mutation to occur in 3.5% in all populations [98]. The global minor allele frequency (MAF) is 27.2% in the 1000Genome phase 1 population (http://www.1000genomes.org) from 1094 individuals from across the world, making it a common polymorphism [99]. In this study, it was detected in both homozygous and heterozygous forms in a total of 31% of the cohort of Egyptian controls. In conclusion, the fact that this variant is present in the homozygous state in controls excludes mostly the possibility of pathogenicity or disease susceptibility.

4.6 Comparison to previous studies

SF-1 mutations have recently been proven by various studies to be a frequent cause of 46, XY DSD with partial gonadal dysgenesis (6.5-15%) leading to the overlapping spectrum from female or ambiguous genitalia to hypospadias [44-46].

This work is the first to be done on an oriental cohort of 46,XY DSD patients without adrenal insufficiency searching for SF-1 mutations in patients from the Middle East and Arab region, revealing a frequency of 4% (2 out of 50). The frequency is in concordance to that of the European region.

European studies reported a frequency of *3-5% NR5A1* mutations in hypospadias patients [44, 45]. All these cases displayed a phenotype of severe penoscrotal hypospadias and undescended testes [45] with the exception of one patient who had simple distal penile hypospadias [44]. Our study revealed a frequency of 8.5% in the hypospadias group. We detected two novel *NR5A1* mutations with reduced transcriptional function in 23 patients with hypospadias (8.5%). This rather high frequency might be due to preselection of more severe cases. The first study of *NR5A1* mutations in a large cohort of hypospadias patients involved 60 children with a range of nonsyndromic hypospadias from the German DSD network [45]. Novel heterozygous

SF-1 (*NR5A1*) mutations were found in 5% (3/60) of the entire group. The affected cases were 3 children with the most severe phenotype of hypospadias comprising severe penoscrotal hypospadias, small to normal phallus, inguinal testes and low testosterone levels [45]. Of note, these changes were found only in patients with severe penoscrotal hypospadias, and hence the authors recommended screening of *NR5A1* gene in cases with severe hypospadias due to gonadal dysgenesis, but not mild or simple, hypospadias cases. Another study on SF-1 mutations in 46,XY DSD patients reported a case of simple distal penile hypospadias, an incidence of 3% in the 33 hypospadias cases studied [44]. This case of isolated distal hypospadias carried a de novo heterozygous missense mutation (p.Arg313Cys) located in the ligand-binding domain of the *NR5A1* gene [44]. Warman et al. 2011 reported a family with 6 members harboring a heterozygous *NR5A1* hinge region nonsense mutation (p.Tyr183X) and displaying a wide range of different phenotypes, including isolated hypospadias, 2 family members with severe perineal hypospadias and one with mild glandular hypospadias [49].

In 46,XY DSD patients with severe underandrogenisation phenotype (severely ambiguous genitalia due to partial gonadal dysgenesis or complete female external genitalia due to complete gonadal dysgenesis), a frequency of *NR5A1* mutations of 8-15% has been reported in Europe [44-46]. In this study no mutations were found in a similar phenotype. The reason for this might be the small number (13) of investigated patients in this subgroup.

4.7 SF-1 – recommendations

This study detected SF-1 mutations with reduced transcriptional activity in 4% of 50 46,XY DSD patients from an oriental cohort. The study forms part of the growing body of evidence showing that up to 10% of 46,XY DSD patients will have a mutation in *NR5A1* and highlights the importance of collaborative efforts aimed at increasing the knowledge about the occurrence of SF-1 in DSD. It is certainly of help for the diagnosis and management to include the *NR5A1* gene in the molecular genetic work-up for those patients. Therefore it is recommended to screen for *NR5A1* mutations in 46,XY DSD patients with severe and milder forms of underandrogenization due to gonadal dysgenesis. Regular monitoring of gonadal function in adolescence and adulthood in

males with *NR5A1* mutations is strongly recommended, as it has been previously reported that SF-1 mutations can cause a progressive disease with deterioration in the gonadal function and impaired fertility with advancing age [52, 78]. Early cryoconservation of detected sperms in attempt to preserve future chances of fertility should be considered. To date, only one study has reported germ cell malignancy in a patient with *NR5A1* mutation [100]. Thus, the gonadal tumor risk of patients with SF-1 mutations seems to be rather low. But further studies are needed to estimate tumor risk in patients with SF-1 mutations.

5 Summary

A growing number of heterozygous mutations in *NR5A1* have been identified in patients displaying the most common SF-1 phenotype of 46,XY DSD with ambiguous genitalia due to partial gonadal dysgenesis at birth [46, 47, 49, 77, 85-89]. Recent studies have reported several cases with *NR5A1* mutations and with the milder 46,XY DSD phenotype of hypospadias due to partial gonadal dysgenesis [44-46, 49]. This case-control study reports three novel *NR5A1* mutations (p.Arg62Cys, p.Glu121AlafsX25 and p.Ala154Thr) with variable degrees of hypospadias from an Egyptian cohort of patients with 46,XY DSD. But, only 2 of the detected mutations are considered to be functionally relevant, as they caused reduced transcriptional activity of the SF-1 protein.

An estimated frequency of 3-5% has been reported in patients with hypospadias in Europe [44, 45]. In this study novel SF-1 mutations with reduced transcriptional activity were found in 8% of patients with hypospadias (2 of 23 cases). This is the first work searching for SF-1 mutations in patients from the Middle East and Arab region, which suggests that the frequency of SF-1 mutations in hypospadias due to gonadal dysgenesis there is the same as in European cohorts.

In conclusion, due to the risk of future fertility problems, the screening of *NR5A1* in patients with hypospadias and gonadal dysgenesis as well as yearly follow-ups of gonadal function is recommended starting at puberty. But also XY patients with ambiguous to female external genitalia due to severe gonadal dysgenesis should be screened for *NR5A1* to elucidate the genetic cause. Elucidating the genetic cause in XY DSD patients might be helpful for future recommendations concerning the so far unclear gonadal tumor risk and possible fertility.

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7 Appendix

7.1 List of abbreviations

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°C Degree Celsius

µg Microgram

µl Microliter

ACTH Adrenocorticotropic hormone

AMH Anti Mullerian hormone

bp Base pairs

cDNA Complementary DNA

Da Daltons

dH2O distilled water

DNA Deoxyribonucleic acid

dNTP(s) Deoxynucleotide triphosphate(s)

DSD Disorders of sex development

FSH Follicle stimulating hormone

H2O Water

hCG Human chorionic gonadotropin

KO Knockout

LH Luteinizing hormone

LHRH Luteinizing hormone-releasing hormone

MAF Minor allele frequency

MIS Müllerian inhibitory substance

NR5A1 Nuclear receptor subfamily 5, group A, member 1

PCR Polymerase chain reaction

pH pH value

POF Premature ovarian failure

SDS standard deviation score

SF-1 Steroidogenic factor 1

SNP Single nucleotide polymorphism

7 Appendix

T Testosterone

VMH Ventro medial hypothalamic nucleus

7.2 List of tables

7.3 List of figures

Anteilserklärung an etwaigen erfolgten Publikationen

Frau Sally Tantawy hatte folgenden Anteil an den folgenden Publikationen:

S. Tantawy, I. Mazen, H. Soliman, G. Anwar, A. Atef, M. El-Gammal, A. El-Kotoury, M. Mekkawy, A. Torky, A. Rudolf, P. Schrumpf, A. Gruters, *et al.* **Analysis of the gene coding for steroidogenic factor 1 (SF1, NR5A1) in a cohort of 50 Egyptian patients with 46, XY disorders of sex development,** Eur. J. Endocrinol., 170 (2014), pp. 759–767

Autoren:
Sally Tantawy, Inas Mazen, Hala Soliman, Ghada Anwar, Abeer Atef, Mona El-Gammal, Ahmed El-Kotoury, Mona Mekkawy, Ahmad Torky, Agnes Rudolf, Pamela Schrumpf, Annette Grüters, Heiko Krude, Marie-Charlotte Dumargne, Rebekka Astudillo, Anu Bashamboo, Heike Biebermann und Birgit Köhler

Titel:
‘Analysis of the gene coding for steroidogenic factor 1 (SF1, NR5A1) in a cohort of 50 Egyptian patients with 46,XY disorders of sex development’

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Unterschrift der Doktorandin

Eidesstattliche Versicherung

„Ich, Sally Tantawy, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: [**Untersuchung des Steroidogenic Factor 1 kodierenden Gens *NR5A1* in einer Kohorte von 50 ägyptischen Patienten mit 46,XY Störungen der Geschlechtsentwicklung**] selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.
Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

7 Appendix

7 Appendix

Publikationsliste

- Tantawy S, Mazen I, Soliman H, Anwar G, Atef A, El-Gammal M, El-Kotoury A, Mekkawy M, Torky A, Rudolf A, Schrumpf P, Grüters A, Krude H, Dumargne MC, Astudillo R, Bashamboo A, Biebermann H, Köhler B, *Analysis of the gene coding for steroidogenic factor 1 (SF1, NR5A1) in a cohort of 50 Egyptian patients with 46,XY disorders of sex development.* Eur J Endocrinol, 2014. **170**(5): p. 759-67.

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