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Androgen insensitivity syndrome

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Abstract. – OBJECTIVE: We provide a review of the literature about the Androgen Insensitivity Syndrome (AIS), its onset and associated developmental anomalies and the genetic alterations causing it.

MATERIALS AND METHODS: We searched PubMed with a larger emphasis on the physiology, genetics and current management of AIS.

RESULTS: AIS is an X-linked recessive Disorder of Sex Development (DSD). It is caused by mutations of the Androgen Receptor, and their large amount and heterogeneity (missense and nonsense mutations, splicing variants, deletions, and insertions) are responsible for the wide spectrum of possible phenotypes of patients, divided into Partial AIS (PAIS) and Complete AIS (CAIS). Once the clinical and laboratory investigations have laid the foundation for a diagnostic hypothesis, it is important to identify the actual karyotype of the individual and search for the mutation in the Androgen Receptor to diagnose with certainty the syndrome. Alternatively, in the absence of such evidence, the diagnosis should more properly be an AIS-like condition, which we describe as well in our report.

CONCLUSIONS: The management of this DSD is based on pharmacotherapies, surgery and psychological support: all of them must be directed to facilitate the patient's life, considering his/her sexual identity.

Key Words:
AIS, PAIS, CAIS, AIS-like, Androgen Receptor, Disorders of Sex Development (DSD).

Introduction

In animals, the development of a new individual organism starts with fertilization, the fusion of an ovum and a sperm, to create the diploid zygote. Subsequent mitotic divisions allow the formation of the specific, recognizable stages of blastula, gastrula, and then organogenesis, finally resulting in the development of an embryo. The fusion of gametes defines the genetic pattern of the individual, sex determination included. If the embryo presents a 46 XY karyotype, under normal circumstances it heads toward the formation of a male foetus because of the presence of the testis determining factor (TDF) on the Y chromosome[1].

The Genetics of Foetus Development

The initiation of male sex characterization in humans is regulated by the Sex-determining Region Y gene (SRY, situated on Yp11.2). This gene encodes a transcription factor that is a member of the high mobility group (HMG) box family of DNA-binding proteins. The encoded protein is the Testis-Determining Factor (TDF), which initiates male sex determination (Figure 1). Mutations impairing SRY function cause sex reversal, with females with an XY karyotype and affected by the gonadal dysgenesis syndrome[2]. Transposition of part of the Y chromosome containing this gene to an X chromosome, either by unequal

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X-Y crossing over during male meiosis or by chromosome translocation, causes the birth of males with an XX karyotype and affected by the XX male syndrome (also called *de la Chapelle syndrome*)[3] (Figure 1). A summary of the main genetic events regulating the early steps of sex determination is illustrated in Figure 2. The fundamental and specific task of the TDF consists in the activation of a male-specific transcription factor, belonging to the family of DNA-binding proteins, called SRY box 9 (SOX9, encoded by a member of the SRY box gene family on 17q24)[4] and the Steroidogenic Factor I (SF1, encoded by the NR5A1 gene on 9q33)[5]. SOX9 is a protein coding gene that directs the male pathway in the foetus and plays an important role also in the normal skeletal development. Therefore, mutations of this gene are often associated with skeletal dysplasia, with campomelic dysplasia, and with genital ambiguity[6].

The gene NR5A1 (Nuclear Receptor Subfamily 5 Group A Member 1) encodes for the Steroidogenic Factor 1, a transcriptional activator essential for sexual differentiation and formation of the primary steroidogenic tissues. Mutations involving this gene are largely considered a major cause of human Disorder of Sex Development (DSD)[7].

SOX9 and SF1 proteins allow the embryonic cells of the primordial gonads to start their differentiation into Sertoli cells at around day 50 after fertilization, determining the making of a primordial testis. Simultaneously, SOX9 and SF1 suppress sexual female characteristics, through the regulation of the production of the anti-Müllerian hormone (AMH, produced by Sertoli cells), a member of the transforming growth factor-beta gene family, which mediates male sexual differentiation (Figure 2). This hormone has the ability to inhibit the development of the Müllerian ducts (which would otherwise differentiate into the uterus and fallopian tubes) in male embryos, promoting instead the making of Wolffian ducts.

Sertoli cells contribute to testicular embryogenesis also by producing a protein called Desert Hedgehog (DHH, located on 12q13), whose role is to promote the maturation of Leydig cells in the making of primordial testes (Figure 2). DHH is a member of the Hedgehog gene family encoding signalling molecules that play an important role in regulating morphogenesis. Defects in this protein have been associated with 46,XY partial gonadal dysgenesis[8].

Another gene involved in the male reproductive system's development is Fibroblast Growth

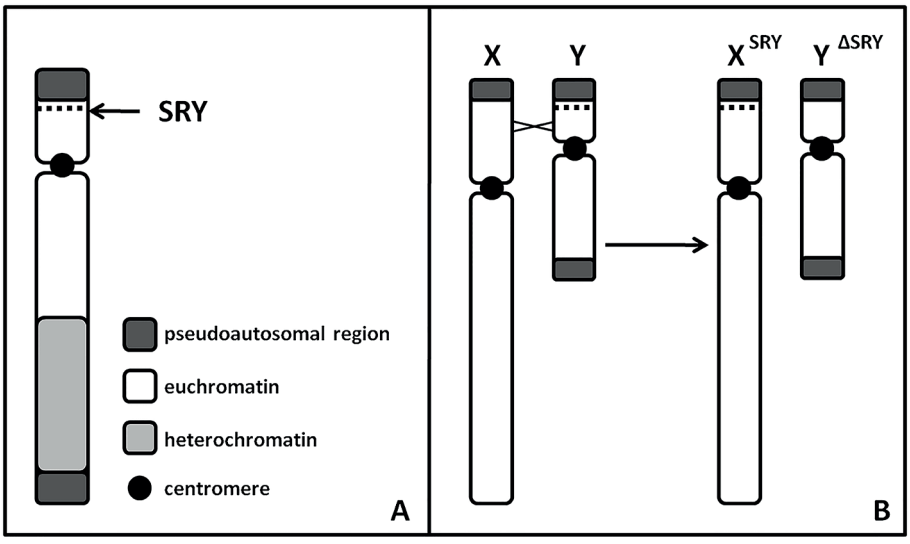

Figure 1. Sex determination in *Homo sapiens* depends on the dominant effect of the Sex-determining Region Y (SRY) gene, coding for the Testis Determining Factor (TDF) mapping in the short arm of the Y chromosome. ***A,*** The Y chromosome has two regions of homology with the X chromosome (dark grey; pseudoautosomal region) and a male-specific region (white, light grey) containing, among the others, the SRY gene. ***B,*** As a consequence of an aberrant crossing over (thin crossed lines) involving the male specific region, the SRY gene may translocate to the X chromosome. Thus, the translocated X chromosome (X^{SRY}) may induce a male phenotype in a person with a 46,XX karyotype, while the translocated Y chromosome ($Y^{\Delta SRY}$) may cause a female phenotype in a person with a 46,XY karyotype.

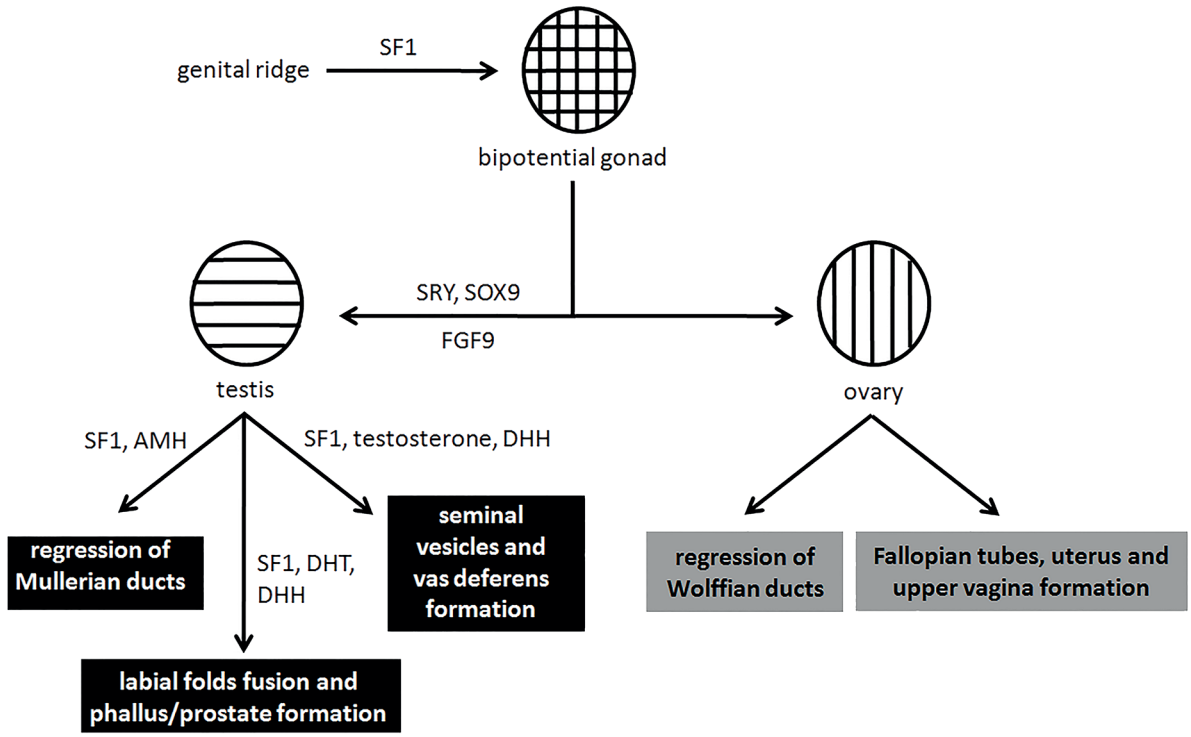

Figure 2. Summary of the main genetic events regulating the early steps of sex determination. The dominant effect of TDF is sufficient to drive the bipotential gonad towards a male differentiation. See the text for detailed explanations.

Factor 9 (FGF9, located on 13q11-12) (Figure 2) encoding a protein, which is member of the fibroblast growth factor (FGF) family. FGF9 carries out many biological processes, including embryo development, cell growth and testicular embryogenesis[9]; therefore, FGF9 is activated by the presence of SOX9 and simultaneously increases the expression of both genes, determining a positive feedback with SOX9 itself[10]. Presumably, FGF9 performs basic processes in human testicular embryogenesis; indeed, mice lacking this gene display a male-to-female sex reversal phenotype[11].

Instead, an embryo with a 46,XX karyotype has a typical female development (Figure 2). The absence of the Y chromosome implies the lack of the *SRY* gene and, consequently, of the TDF; for this reason, the bipotential cells of embryonic gonads develop into ovaries, the Wolffian ducts atrophy, and the Müllerian ducts develop into the uterus, Fallopian tubes, cervix and upper portion of the vagina. Furthermore, because ovaries do not produce androgens, the proto-phallus develops as a clitoris, the labioscrotal folds become the labia, and the urethra maintains a female-typical position[12]. At puberty, the sexual differentiation is maintained by the estrogens, which are produced by the ovaries[12].

Androgen Insensitivity Syndrome - AIS

AIS is one of the most commonly diagnosed XY DSD, with an estimated prevalence of 2:100.000 to 5:100.000[13] and an incidence of 1:20.000[14] to 1:99.000[15]. It consists in the partial or complete inability of the cell to respond to androgens[16]: this cellular inability can lead to the wrong development of primary and secondary sexual characteristics. Historically, AIS is known with several different names; here we remember for example Reifenstein syndrome[17], Goldberg-Maxwell syndrome[18], Morris' syndrome[19], Gilbert-Dreyfus syndrome[20], Lubs' syndrome[21], incomplete testicular feminization[22], Rosewater syndrome[23], and Aiman's syndrome[24]. The different names reflected differences in patients' phenotypes, thus inducing scientists and physicians to believe that they were due to different etiologies. Only later, the analysis of families with recurrent pathologies but different phenotypes and the advancement of molecular characterization of DSDs revealed that the pathogenesis of AIS is characterized by different phenotypic expressions of molecular defects in the Androgen Receptor (AR) coding gene and that the different phenotypes reflected just the different mutations of the AR sequence alone[25].

In conclusion, AIS is caused by a mutation of the gene that provides information for the making of the AR (also known as NR3C4)[26] (Figure 3). AR is a nuclear receptor that allows cells of many tissues to respond to androgens, by binding testosterone and dihydrotestosterone (DHT). The wrong dimeric assembly of this receptor brings to a variable grade of insensibility of the cells towards androgens[27] from a lower to a higher grade of severity. In the most severe cases, the mutations completely abolish AR dimerization. Ultimately, the different mutant phenotypes of AIS patients mirror the different strengths of hormone/receptor affinity. Androgenic hormones play a crucial role in several stages of male development, such as sexual differentiation, initiation and maintenance of spermatogenesis and of secondary male features, feedback regulation of gonadotrophin secretion[28]. The interaction between the hormone and its androgen receptor produces a direct regulation of gene expression, promoting the correct development of male characteristics in a 46,XY foetus[29].

In AIS, the presence of the Y chromosome implies the normal activity of the SRY protein and finally the creation of male gonads. However, the inability of the foetal cells to properly respond to androgenic hormones causes the wrong development of the male reproductive system; therefore, the testes are often retained in the abdomen or, in some cases, they are situated in the labia majora (if they develop as such) or in different tracts of the migration ducts[30]. Consequently, the suppression of the development of female sexual characteristics (regulated by SOX9 and SF1, which control the activity of the anti-Müllerian hormone) (Figure 2) is no longer present. These events can lead to the formation of a full female habitus, especially when the androgenic unresponsiveness is particularly severe or complete, despite the 46,XY karyotype of the individual.

Clinically, it is possible to distinguish different phenotypes of this disorder. The most moderate form of AIS can manifest in a normal male habitus with mild spermatogenesis defect or reduced secondary terminal hair. There is also an intermediate form in which the external genitalia are not fully masculinized, but the androgen insensitivity is not strong enough to define a complete feminization of the individual. Finally, there is the most serious form in which the androgen insensibility is complete and, in this case, there is a fully de-

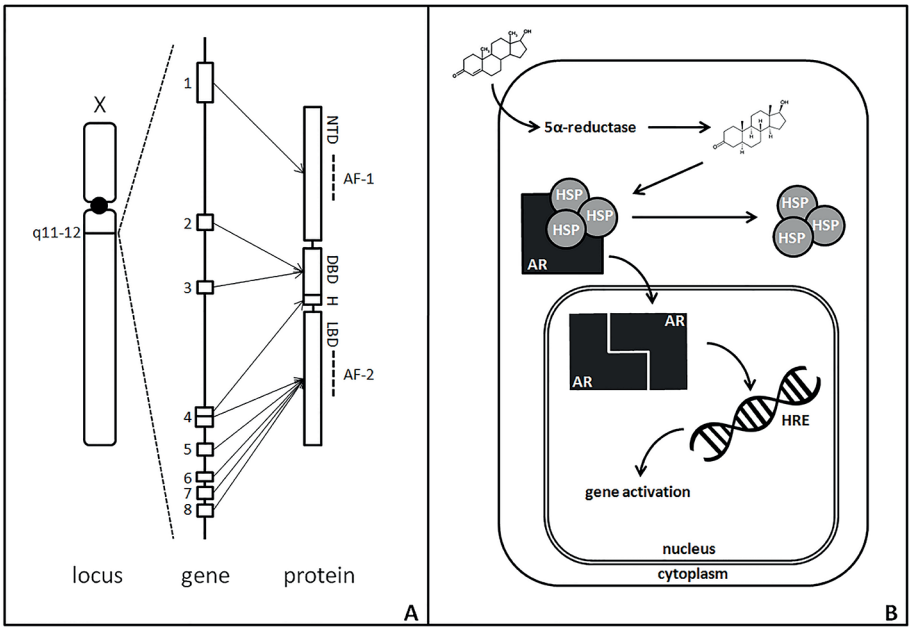

Figure 3. Structure and function of the Androgen Receptor gene. ***A,*** From left to right: position of the locus on the X chromosome; AR gene organization (8 exons, numbered 1-8); AR protein organization, with the indication of domains and their correlation with exons (see arrows). In the gene, exon 4 has a subdivision since it partly encodes the hinge domain and partly encodes the first part of the ligand binding domain. In the protein, thin lines indicate linker sequences between domains; NTD: N-terminal transactivation domain; DBD: DNA binding domain; H: hinge domain; LBD: ligand binding domain. Dotted vertical lines indicate activation functions AF1 and AF2. ***B,*** Mechanism of AR activation upon DHT binding. See the text for detailed explanations.

veloped feminine phenotype with the presence of normal external genitalia such as labia, clitoris and a vaginal depth, even if it is typically shorter than normal ones[31]. The absence of uterus and ovaries causes primary amenorrhea, which is the main and first symptom to diagnose this disorder. Thus, experts distinguish two main categories of AIS: 1) Partial Androgenic Insensitivity Syndrome, or PAIS; 2) Complete Androgenic Insensitivity Syndrome or CAIS. Only the latter is responsible for the fully developed external female reproductive system in patients with a 46,XY karyotype[32].

Partial Androgen Insensitivity Syndrome - PAIS

PAIS is a DSD that results in the partial inability of the cell to respond to androgens. This condition is generally due to missense mutations in the androgen receptor gene and causes the mildest forms of AIS[33]. PAIS patients display a fully developed male reproductive system, but often associated with severe hypospadias, micropenis, bifid scrotum (in which the testes may or may not descend) and infertility, due to presence of a minimal defect in the androgen receptors[34]. The more serious clinical conditions caused by PAIS can show progressively lower grades of male differentiation of external sexual organs, very frequently resulting in the presence of ambiguous genitalia at birth (for example, an enlarged clitoris) and pubertal undervirilization, sometimes characterized by gynecomastia, decreased secondary terminal hair and high-pitched voice[35]. The diagnosis in childhood may be difficult: it is necessary to find what type of mutation of the androgen gene is responsible for the disorder and, in some cases, it is not possible to find one in the coding sequence[36]. In puberty and adult age, the diagnosis is based on clinical and biochemical findings in XY patients with varying degrees of under-masculinization: the typical hormone profile shows increased luteinizing hormone (LH) and testosterone levels[37]. Determination of testosterone, testosterone precursors and DHT levels at both baseline and after human chorionic gonadotropin (hCG) stimulation should be performed to exclude an androgen biosynthetic defect[38]. Management of PAIS is complex, especially when patient sexual identity is unclear. Current guidelines report that parents and healthcare professionals should give gender assignment in borderline individuals as early as possible in infancy, avoiding waiting for the child to decide[39]. This assignment should be done considering the appearance of external genitalia, child's virilisation capacity, complexity of genitoplasty, chances of gaining fertility and the projected gender identity of the child[13]. It is probable that the more virilised the genitalia are, the more likely the brain has been masculinized[40]. Those children raised as males require chirurgical hypospadias repair, orchiopexy for undescended testes, reduction mammoplasty in case of gynecomastia and large doses of androgens to induce puberty[13], which, in combination with intracytoplasmic sperm microinjection, can rarely restore fertility even in the mildest cases[41]. Those patients raised as females need gonadectomy before puberty to avoid the possible further virilisation and to prevent the very high chances of malignant transformation of undescended testes[42]; in addition, they require puberty induction with estrogen hormones treatment[43]. In both cases, psychological support from birth to adulthood is necessary to give the patient awareness and help him/her live with this condition.

Complete Androgen Insensitivity Syndrome - CAIS

CAIS is characterized by the presence of female external genitalia in a 46,XY individual with normal testis development but undescended testes, due to complete unresponsiveness of the cells toward androgens[14]. The presence of the SRY region promotes the formation of primordial testes in the foetal abdomen and, by the 7th week after conception, foetal testes begin to produce testosterone, whose activity is blocked by the pathological processes affecting ARs. The CAIS phenotype is associated with an *AR* gene mutation that completely disrupts receptor's function; target cells do not respond to testosterone or DHT. An *AR* gene mutation is found in more than 95% of patients with CAIS, 70% of them being inherited and 30% *de novo* mutations[31]. This androgen insensitivity is diagnosed in 1/20.000 live male birth[14] and allows the estrogen to take over; hence, the female appearance and development of female external genitalia. However, the presence of the anti-Müllerian hormone, produced by primordial testis, suppresses the formation of female genital organs. The lower part of the vagina, instead, is completely developed because it is not a Müllerian duct derivative; however, it is shorter than normal and presents a blind ending[44]. The complete absence of any sign of masculinization in external genitalia and of any typical secondary masculine characteristic is the reason why these individuals are always raised as females, have a female gender

identity, and are anatomically and legally women. As a consequence of patient's apparently normal female habitus, CAIS is very rarely diagnosed in childhood. The typical presentation of this syndrome is during puberty in the form of primary amenorrhea. Individuals with CAIS reach puberty at a later stage than other girls[14]: the hypothalamus and the pituitary gland continue to stimulate testes to produce testosterone, which is converted to estradiol[14]. The presence of estradiol, in addition to the insensitivity to testosterone, promotes the formation of typical female features in puberty: there are normal breast development, normal reshaping of the pelvis, redistribution of body fat, little or no appearance of pubic or other androgenic hair and rare appearance of facial acne[14]. A feature that is more frequently observed in individuals with CAIS is the slightly increased height compared to average females; the reason for tallness appears to be the presence of the Y chromosome, which may have an effect on growth, independently of hormonal changes[14]. During childhood, no treatment is needed because these patients have normal female hormonal levels[31]. Successful management of CAIS patients requires gonadectomy, vaginal enlargement, estrogen replacement, and genetic counselling[33]. Gonadectomy is usually recommended only in early adulthood[45] because the testosterone produced by the testes is converted to estrogen in the body tissues and that entails an advantage - in this way pubertal changes will happen naturally, without hormone replacement. Gonadectomy is usually suggested because undescended testicular tissue presents increased risk of malignant transformation after puberty. The best evidence suggests that women with CAIS and PAIS retaining their testes after puberty have a 25% chance of developing benign tumours and a 4-9% chance of malignancy[46]. For the treatment of vaginal hypoplasia, dilation should be the first approach[14]. Dilation is performed by applying pressure to expand the tissue over an extended period of time. Plastic surgical techniques to construct a new vagina from donor sites should only be resorted to, once the dilation is ruled out[14]. One of the most important measures to implement is the planning of psychological support because of the distress that AIS produces in patients[47].

The Genetics of AIS – AR Gene Structure

AIS is an X-linked recessive disorder caused by mutations of the AR gene, (cytogenetic location: Xq11-12)[48]. Generally, 46,XY individuals with a mutated AR gene are sterile and unable to transmit it to the progeny[14]. However, a scarce minority of persons affected by the mildest form of PAIS, are either fertile or may become fertile with the use of supplementary testosterone[41]. Female descendants of these individuals will inherit a copy of the mutated AR gene, and thus become carriers. These females, in turn, will have a 50% chance to transmit the mutated gene to their children. In theory, the XX progeny of a fertile male with very mild PAIS and of a female carrier may be a 46,XX person with both mutated AR receptor genes, but this is a very unlikely occurrence.

The AR gene-coding region consists of 920 amino acid sequence (≈99 kDa) (NCBI reference sequence NM_000044.2) (Figure 3). It is organized in 8 exons. The protein contains three major functional domains: (1) the N-terminal domain, a transactivation domain, which starts the transcription of the target genes; (2) the DNA-binding domain (DBD), which interacts with DNA and is essential to bind the hormone response elements (HREs); (3) the ligand-binding domain (LBD), which promotes first the interaction of the receptor with the HSPs in the cytoplasm and, then, with the androgen hormone, leading to the migration of the AR to the nucleus. Specifically, the transactivation domain is more than a half of the receptor, is entirely encoded by exon 1 and spans residues 1 to 534; exons 2 and 3 encode the DBD, from residue 559 to 624; the proximal portion of exon 4 encodes for the Hinge domain (residues 629 to 634), located between the DBD and the LBD and whose role is to contribute controlling the AR activity; the remaining part of exon 4 and exons 5-8 encode for the LBD, which includes residues 664-920. The androgen-signaling pathway consists also in many steps and biochemical interactions regulated by several types of cofactors and coactivators. Two of them play a crucial role for the correct development of androgen binding and the receptor activity: they are activation function 1 (AF-1, inside the N-terminal domain) and activation function 2 (AF-2, inside the C-terminal LBD) (Figure 3).

The androgen receptor protein, also known as the DHT receptor or NR3C4, belongs to the family of nuclear receptors that are typically located in the cytoplasm where, in baseline conditions, the androgen receptor forms a multimeric complex with the Heat Shock Proteins (HSPs), especially with HSP90, HSP70 and HSP56)[49] (Figure 3). When the androgen hormone reaches the cytoplasm, it causes the dissociation between the AR and the HSPs, binds to the AR itself and causes the

migration of this new complex inside the nucleus[50]. At that point the androgen receptor dimerizes and then stimulates the transcription of androgen responsive genes by binding HREs[51] (Figure 3).

The Genetics of AIS – the Effects of Gene Deletion

Several types of mutations in the AR gene cause AIS: from complete and partial gene deletions, to point mutations, to small insertions or deletions. The very high number of possible mutations of the AR gene is responsible for the clinical heterogeneity of the syndrome, depending on the quantity and quality of the remaining receptors. For example, a complete loss of receptor function in the cytoplasm may be caused by a wrong or incomplete protein synthesis; otherwise, mutations that cause altered substrate-binding affinity can produce a signal transmission loss, despite a normal cell receptor number[52]. Therefore, there are four different types of mutations in this gene: (1) point mutations, which can lead to a single amino acid substitution or to the placement of a premature stop codon; (2) frameshift mutations, caused by nucleotide insertions or deletions; (3) complete or partial gene deletions, mostly causing a complete deficit of the protein; (4) intronic mutations in either splice donor or acceptor sites[53].

In some cases, complete or gross deletions of the AR gene have been reported[54,55]; this type of mutation generates a complete insensitivity toward androgen hormones. Even though these deletions occur quite rarely, they mostly cause the development of a CAIS phenotype, due to the complete loss of the AR protein. That is a peculiar difference between complete deletions and other types of mutations of the AR gene, in which we can usually find some receptor proteins, even if they may be not functional[54]. However, gross or complete deletions of the AR gene are only a minimal part of the possible molecular etiologies of AIS, most of all because of their very infrequent prevalence in patients. To make the research and the updating of new mutations easier, it is useful to rely on the AR gene mutations database, available at http://androgendb.mcgill.ca/[56] (a detailed map of mutations is available at: http://androgendb.mcgill.ca/map.gif). Notably, the AR mutations database is currently updated to September 2014, thus novel mutations discovered after this date are not available in the database. Furthermore, it is necessary to verify the actual position and nomenclature of mutations detected in every study published before the publication of the database, since there is the possibility that the progressively improved quality of technologies in genetics may have caused the variation of some of them.

The Genetics of AIS – Mutations in the Transactivation Domain of the AR Gene

Some portions of the *AR* gene are more prone to mutate than others: in fact, the frequency of mutations in exon 1 is relatively low, if compared to the number of mutations detected in exons from 2 to 8[57]. Two different clusters in exons 5 and 7 show a higher number of mutations, while very few mutations were discovered causing AIS in the first portion of exon 4, encoding the Hinge region[58].

Exon 1 of AR is subject to mutations that may cause either CAIS or PAIS phenotypes. For example, single nucleotide insertions or deletions cause frameshifts in exon 1, determining the creation of premature stop codons. A cytidine insertion in codon 42 is responsible for the formation of a premature stop codon at position 171; similarly, an adenine deletion in codon 263 produces a premature stop codon at position 292. Both mutations cause CAIS - in fact, no AR protein expression was detected[58]. Recently, two more mutations in this region have been identified, due to 1 and 4 nucleotides deletion respectively; in both cases, the patients display a CAIS phenotype as a consequence of a premature stop codon causing the production of a truncated protein[59]. CAIS individuals are also carriers of other types of mutations: for example, a missense mutation inside a tyrosine codon at position 514 caused the formation of a stop codon (Y514*) in a couple of Iranian siblings, determining a fully developed feminine phenotype[60]. Moreover, a guanine-to-adenine transition at codon 503 was discovered in a CAIS patient, converting a tryptophan into a stop codon (W503*)[61]. Another point mutation that has recently been identified consists in the substitution of a valine with a methionine in position 30 (V30M), detected in a CAIS patient[62]. A frameshift mutation (c.118delA) - first discovered in two sisters diagnosed with CAIS during childhood - causes the wrong assembly of a truncated (173 aminoacids long) receptor[63]. The insertion of an adenine at position 60 in the polymorphic CAG trinucleotide region produces a truncated protein, with the creation of a premature stop codon at position 83, determining a CAIS phenotype[64]. The polymorphic CAG sequence length ranges from about 8 to 31 repeats, averages about 20[65] and encodes for a polyglutamine chain. The variable length of this polymorphic region has been associated

with different hormonal disorders: a progressive expansion of CAG repeats causes a linear decrease of transactivation function in the AR. Hence, the shorter this sequence, the higher the transcriptional activity of the AR, strongly suggesting that the polyglutamine chain is inhibitory for transactivation[66]. Therefore, an enlarged CAG sequence is commonly associated with AIS, mostly when it reaches an extremely large size (≥40 repeats), often causing also spinobulbar muscular atrophy or Kennedy syndrome. On the other hand, a too short CAG repeat sequence implies a higher risk of prostate cancer[67]. Exon 1 is also subject to mutations that are responsible for different phenotypical patterns. For example, the missense mutation R407S causes PAIS by creating a phosphorylation site which inhibits the action of melanoma antigen-A11 (MAGE-11, an essential coregulator for proper AR function)[68]. Another PAIS phenotype is caused by the missense mutation A242S, which has been linked also to azoospermia and cryptorchidism in a 49-year-old man[69]. A further case of PAIS has been diagnosed by discovering a missense mutation in codon 176 of exon 1 (S176R), which is predicted to cause damages to both structure and function of the protein[70].

The Genetics of AIS – Mutations in the Dna Binding Domain of the AR Gene

Over the past years, several studies focused on pointing out the possible mutations that exons 2 and 3, encoding the DBD, could be hit by. These studies reported a relatively high number of mutations that can damage the structure and function of the domain; most mutations cause CAIS. DBD presents two peculiar zinc finger modules necessary to bind the DNA and stabilize the protein (Figure 3). Their structure is dependent on cysteine interactions with a zinc ion that play a fundamental role in the correct function of the receptor. In case of mutated cysteines, the activity of zinc fingers is compromised, impairing the whole domain function and determining a CAIS phenotype, even if the binding ability of the receptor for androgen hormones is still present[33]. Codon 616 (CGT) belongs to the second zinc finger and encodes for an arginine and fifteen substitutions and one deletion have been reported for this codon. One of them is R616C, which causes CAIS in a Chinese XY patient, belonging to a family with six-affected individuals[71]. A nucleotide substitution (G---C) within the second zinc finger (R616P) determines a functional receptor failure and CAIS[72]. The same 616 codon is the site of another mutation found in two siblings consisting in a single base substitution (G---A) and the replacement of the arginine by a histidine (R616H), making the AR non-functional[73]. A novel missense mutation in the exon 3, caused by a single nucleotide transition (C to A), determined an amino acid interchange between the arginine and a serine at the same 616-codon position (R616S), resulting in CAIS affecting three of four daughters of a heterozygous mother[74]. In addition, a single codon 616 deletion has been identified causing CAIS[75]. The DBD can be subject to many other different types of mutations. For example, a deletion of the exon 2 has been found in a young Chinese with a fully developed external female reproductive system, with normal breasts, vellus hair in the armpit area but with no pubic hair and a blind-ending vagina. This deletion involves the entire exon 2[76]. An Y571C change has been related to CAIS where the target AR gene expression is decreased, making the patient completely resistant to androgens[77]. The deletion of one of the two adjacent phenylalanine residues in codon 583 and 584 leads to CAIS as well[75]. A frameshift mutation was found in a 22 years old 46,XY female, who also presented a gonadoblastoma in one gonad and a gonadoblastoma with dysgerminoma in the contralateral gonad, caused by undescended testes. The five nucleotides "AGGAA" deletion from nucleotide 2940 to 2944 (codons 609 and 610), resulted in missing arginine and lysine followed by a premature stop codon at position 621[78]. Another AIS patient showed a point mutation of a donor splice site in intron 2 with skipping of the entire exon 2 and a premature stop codon at position 599 resulting in a truncated and non-functional AR in a patient with a completely female phenotype[79].

The Genetics of AIS – Mutations in the Ligand Binding Domain of the AR Gene

The first half of exon 4 encodes the hinge region, a small domain between the DBD and the LBD, which is associated with phosphorylation, acetylation, and degradation of AR protein[80]; to the best of our knowledge, only four mutations have been described in this region[56,81]. The LBD is encoded by the remaining part of the exon 4 and by exons 5-8. It is fundamental for the receptor function, permitting AR to bind androgens. Several types of mutations can be found in this domain. They are: complete deletions of exons 3-8[82] or exons 4-8[83], with a partial AR loss in 46,XY females; single nucleotide deletions, such as the adenine in exon 4 leading to a premature stop codon (K638*) and CAIS[84]; amino

acids substitutions, duplications and insertions. Some LBD portions are more likely hit by mutations: they are usually found between aminoacidic residues 688-712, 739-784, and 827-870[33,56]. We here mention some of the many mutations in the LBD that have been discovered. The G>T substitution at position 689 results in the GGA---TGA change and determines the formation of a premature stop codon instead of a glycine[85]. Another nonsense mutation was found in a family with three affected siblings who showed a 46,XY karyotype with a fully developed female phenotype, primary amenorrhea, clinical and endocrinological signs and symptoms referable to AIS: in those cases, a G>A mutation in codon 717 caused the change of the tryptophan in a stop codon, making a non-functional receptor[86]. Point mutations such as P683T, Q712E and D880Y have been included in the possible etiologies of PAIS[86]; G744E, F828V and L831V, instead, are all responsible for the CAIS phenotype; also, a splice junction mutation (G---C at +5, exon6/intron6) has been identified, causing CAIS[87]. A new splice variant, due to the insertion of 5 nucleotides in the junction between exons 6 and 7, has been recently described causing the formation of a new splice acceptor site upstream of the original site, resulting in the introduction of a premature stop codon, thus producing a truncated protein of 823 amino acids[88]. Interestingly, it has been reported that in case of synonymous mutations there is the rare possibility of having an altered AR function, if the affected sequence is also used as a splicing site; such phenomenon has been reported for both PAIS[89,90] and CAIS[91] patients, and may involve any of the 8 exons. Five additional new mutations have been recently described[59] inside exons 6-8 that cause either PAIS or CAIS depending on their role on protein function. A similar situation is described by Petroli and co-workers[92], who report six additional missense mutations in the same exons; interestingly, the functional analysis of these mutated proteins (N/C interaction assay) revealed striking differences among them, not only for the PAIS/CAIS phenotype, but also for the response of the PAIS patients to hormone treatment, further increasing the complexity of this condition.

The Genetics of AIS – Mosaic Individuals

In the cases described so far, in AIS patients all cells are affected by the same mutation. However, sporadically, it is possible to identify patients that are heterozygotes for AR mutations[93,94]; these patients were first described only 25 years ago[95]. To date, only 14 mosaic patients have been described with a mutation in the AR gene[94-97]. In these patients, the mutation occurs at some time after fertilization and then segregates in all daughter cells. As such, the associated phenotype may strikingly change even in the presence of the same mutation, and essentially depends on the time of mutation occurrence – the earlier during development, the worse[98]. For example, the nonsense mutation L172* has been described as causing CAIS[56], but a patient having this same mutation as a mosaic displayed a PAIS phenotype[93]. As expected, the high variability in phenotypes causes mosaic patients to have either PAIS or CAIS, and to be assigned as males or females on a case-by-case basis[94-97]. For the same reason, also the response to hormone therapy is largely unpredictable. No special feature characterizes mosaic mutations – all previously described alterations are present also in these patients, i.e., nonsense and missense mutations, premature stop codons and splicing alterations, distributed along the entire gene length but with a preference for exons 4-8. As for the psychological aspects of AIS mosaic patients, their number reported in the literature is too small to support any strong hypothesis, although the few data available seem to suggest that there are no significant differences in their behavior and in their management, compared to non-mosaic, AIS patients[97].

The Genetics of AIS – Klinefelter Patients

Klinefelter patients are usually phenotypically males that have one extra X-chromosome in their cells[99], consequent to a non-disjunction of the sex chromosomes during parental meioses[100]. This may occur either in the father (failure to segregate X and Y, thus the two X chromosomes in the patient come from both parents) or in the mother (in this case the two sex chromosomes are identical); the chance of this aberrant event is evenly distributed between sexes[101]. To date, only three patients with a XXY karyotype and a non-male habitus have been described in literature. Two of them display a CAIS phenotype[102,103], while the third has the typical PAIS appearance[104]. The two CAIS patients had identical AR mutations, suggesting a meiosis failure in the mother. The PAIS patient was instead heterozygous for both AR gene mutation and Xq, but homozygous for the entire Xp. This is notable, since the identified alteration (the frame shift mutation p.Asn849Lysfs*32 causing a stop codon) was previously described in a patient with CAIS phenotype;

the Authors conclude that the milder PAIS phenotype of the patient is likely due to random X inactivation, an aspect that further complicates AIS etiology and genetics.

AIS-Like Conditions

Even though the androgen-signaling pathway is complex, mutations of the receptor gene are necessary for syndrome diagnosis. This assumption has been challenged by the discovery of several individuals who show typical AIS clinical features, despite the absence of *AR* gene mutations. Studies on these patients show mutations in other genes, for example SRD5A2[105] and 17β-hydroxy-steroid dehydrogenase type 3 (17βHSD3)[106], which can cause a CAIS-like phenotype. Also mutations in steroidogenic factor 1[107] or mastermind-like domain containing 1 genes (MAMLD1, located on the X chromosome, coding for a transcriptional co-activator whose mutations are most commonly associated with hypospadias)[108] are supposed to be responsible for PAIS-like cases. Additionally, AR gene mutations are prevalent in CAIS (about 95% of CAIS patients show an AR mutation), but in PAIS this finding is not so frequent: it has been reported that between 28% and 73% of them (depending on case selection) does not present any *AR* gene mutation[84]. Moreover, no *AR* mutation is discovered in 85-90% of sporadic cases and in 10-15% of familial cases[109]. For these reasons, it is actually possible to find patients with classic clinical patterns of AIS without the typical genetic profile, commonly required to diagnose the syndrome. This kind of diseases are probably caused by mutations in coregulator or coactivator proteins compromising the proper function of the AR, with the development of an ambiguous phenotype that can be easily compared to AIS[110]. Finally, there is another category of patients who cannot be clearly classified: they display a clinical profile and a phenotype that can be assimilated to AIS as well, but without any recognized mutation neither in the AR nor in coregulator or coactivator proteins. In these individuals, it is often recommended a more accurate study of their genetic profile, in order to look for a larger number of possible gene mutations by Next-Generation Sequencing (NGS)[111]. Androgen binding studies performed on cultured fibroblasts may also be performed in selected cases and it is recommended to use foreskin samples, since androgen receptors are more abundant in this area[112]. These tools can be useful to reveal many more details of the factors that can result in a particular phenotype[13].

Conclusions

AIS is a rare DSD, due to mutations of the AR gene, causing variable degrees of resistance to androgen hormones in patients with a 46,XY karyotype. The wide spectrum of possible clinical presentations states the division of patients into two broad categories: patients diagnosed with Complete AIS (CAIS) or Partial AIS (PAIS). CAIS patients display a female phenotype, with fully developed external genitalia and breast, scarcity or absence of axillary and pubic hair, a shorter and blind-ending vaginal depth (due to the absence of the upper portion of the vagina, uterus, fallopian tubes and ovaries) and retained testes in the abdomen or in the inguinal canal. PAIS patients may display a wide spectrum of phenotypes: from predominantly male features (but with impaired spermatogenesis, scarce pubertal virilisation, gynecomastia, hypospadias and either descended or undescended testes), through cases of ambiguous genitalia (gynecomastia, microphallus, bifid scrotum, frequently undescended testes), up to predominantly female clinical patterns (clitoromegaly, posterior labial fusion, inguinal or labial testes). CAIS is usually diagnosed during puberty, when the patient, raised as a female, shows primary amenorrhea; the diagnosis of PAIS may be suspected at birth in patients with ambiguous genitalia. It is important to identify the actual karyotype of the individual and search for mutations in the AR gene, which can range from complete to partial gene deletions, to point mutations, to small insertions or deletions; new mutations are repeatedly discovered worldwide[113-116], including intronic variants causing splicing defects[117,118]. Other diagnostic techniques, such as NGS and androgen binding tests on cultured fibroblasts, are recommended. AIS management depends on the clinical features and gender identity of the individual. CAIS patients are usually subjected to gonadectomy to avoid risk of testicular malignancy; the operation is performed at around 18 years of age because the development of secondary sexual features normally happens due to the aromatisation of androgen hormones produced by retained testes. Consequently, no substitutive hormonal therapy is needed to induce puberty. Otherwise, in case of prepubertal gonadectomy, estrogen replacement therapy is necessary to initiate puberty. PAIS patients, instead, can be treated with different therapeutic objectives, considering the gender identity of the patient: hormonal therapies, surgery and

psychological support must be provided to ensure a comfortable lifestyle.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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