THE NEWBORN WITH AMBIGUOUS GENITALIA. NEW DIAGNOSTIC CONCEPTS. CLINICAL SCENERIES AND DECISIONS. PART 1

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This review contains two parts. Part 1: definition of the problem, description of basic concepts related to normal sexual differentiation, diagnostic orientation for the clinician, and comments on the problem of sex assignment from the personal, social and legal point of views. Part 2 (to be published in the next issue of the Endocrinologia Pediatria On Line Site): description of the most common diagnostic problems to be faced by the pediatrician or the medical team.

THE PROBLEM

A newborn with ambiguous genitalia is a classic urgency in pediatric endocrinology practice [1]. The appropriate management of parents and other relatives anxiety and suffering is the first question to be faced by the clinician. Frequently, the specialist is called after other doctors have made inadequate decisions influencing the whole diagnostic process. Appropriate interrogation is a very useful tool to appreciate, first, parent’s feelings in relation to their fantasies regarding the baby’s sex and the information received so far. Second, the social and familiar context should be evaluated as precisely as possible. After interrogation and initial clinical examination, the doctor decision is expected. In view of the complexity of the problem and the consequences of the announcement, the doctor should be very cautious and, in the majority of cases it is necessary to request some waiting period to complete diagnostic studies, before sex assignment. This waiting period should be as short as possible, but since a multidisciplinary team should participate in the decision, this is difficult to accomplish. Therefore, parents should understand that cytogenetics (occasionally, molecular genetics), hormonal and imaging studies are necessary for the most adequate decision to be taken. At first, it is advisable not to mention the words “testis” or “ovaries”, in the first place because one is seldom sure what kind of gonad is present, palpable or not. A convenient term, as “gonad” can be used after an appropriate explanation. It is also frequent that caryotypes are informed by the geneticist, as “male” or “female”, instead of “46,XY” or “46,XX”, respectively. Strictly, the caryotype is a photograph of nuclear fragments, which some times do not include the expected genes. These terms used prematurely have a profound impact on physicians, parents, and other relatives, and might result in unnecessary psychological damage. Finally, it should always be present that, in some cases, ambiguous genitalia are associated with severe adrenal insufficiency and risk of death.

Frequently, the newborn is referred to Hospital or Health Center of high complexity. Depending on each Center, the medical team is usually composed by a pediatric endocrinologist, a geneticist, a pediatric surgeon specialized in gynecology, a pediatric urology, a psychologist, an imaging specialist, a pediatrician and a layer specialized on sex assignment. We believe that a pediatric endocrinologist should be the team coordinator.
MECHANISMS OF NORMAL HUMAN SEXUAL DIFFERENTIATION

A brief review of the mechanisms of normal human sexual differentiation might be useful to understand the pathogenesis of ambiguous genitalia [2]. Sex determination is defined at the time of fecundation with the formation of an egg with a 46,XY (male determinants) or 46,XX (female determinants) chromosomal constitution.

Figure 1

Figure 1 shows the embryological origin of the main cellular types of the gonads. In the genital ridge, a common support cell precursor is differentiated into precursor Sertoli cells in the testis and precursor granulosa cells in the ovary. An important initial event is the migration of primordial germ cells from the midline to the urogenital ridge on both sides. They differentiate into either 46,XY gonocytes in the male sex or 46,XX gonocytes in the female. A steroidogenic precursor cell is also present in the genital ridge. It is differentiated into either fetal Leydig cells in the testis or theca cells in the ovary. Finally, out the surrounding connective tissue, differentiation of peritubular myoid cell in the testis or perifollicular cells in the ovary takes place. Primordial germ cells proliferate by mitosis during migration. In 46,XY germ cells meiotic division is arrested by an unknown factor, while in 46,XX cells meiotic division is initiated, but it does reach completion.

Testicular differentiation. Initiation of testicular differentiation (7th week of gestation) in the gonadal ridge precedes that of the ovary (10th week). The SRY gene, located in the short arm of chromosome Y plays a central role in testicular differentiation. SRY has only one exon of 612 bp. The SRY protein is expressed in Sertoli and germ cells and participated in testicular differentiation. This transcription factor of 204 aa binds and curves DNA in its mechanism of action. However, it requires the effect of other proteins to differentiate the embryonic testis. This is a complex process which takes place with the participation of multiple proteins. Alterations of one or more of these steps generate different types of gonadal dysgenesis.
The mechanisms of differentiation of non-germ cells in the embryonic testis are shown in Figure 2. The most accepted hypothesis considers that there is a first stage characterized by the differentiation of the bisexual primitive gonad modulated by numerous factors, just a few of them (WT-1, LhX9 and SF-1) are shown in the figure. SF-1, an important factor during embryogenesis and post-natally, is expressed by a gene located in chromosome 9p33. It is necessary for the differentiation of the gonads, adrenals, hypothalamus and pituitary. It also regulates the expression of several enzymes of steroidogenesis, the ACTH receptor, and AMH (anti-müllerian hormone) and its receptor. Recent advances in molecular genetics have help to understand of these processes, but several aspects remain obscure. The SOX9 gene is located in chromosome 17q24, it has 3923 bp and transcribes a mature protein (transcription factor) of 509 aa. It is accepted that, another protein, DAX-1, is an inhibitory factor of SF-1 and SOX9. The DAX-1 gene is located in chromosome Xp21. Furthermore, some evidence suggests that Wnt-4, an extra-cellular growth factor, stimulates DAX-1 and inhibits Leydig cell differentiation. It is important to know that the modulation of these factors is dose-dependent, that is, excessive or insufficient inhibition or stimulation defines the final result. The process of testicular differentiation leads to the differentiation of fetal Leydig, pre-Sertoli and peritubular myoid cells. The latter surround pre-Sertoli cells and gonocytes to form the seminiferous cords. Fetal Leydig cells secrete testosterone, necessary to differentiate gonadal ducts and external genitalia, and Insl-3 (insulin-like 3) which participates in testicular descent. Finally, pre-Sertoli cells secrete AMH to inhibit ipsilateral müllerian ducts to block the formation of uterus and fallopian tubes.

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**Ovarian differentiation.** The delay in ovarian differentiation would be a consequence of the presence of a double dose of chromosome X. Little is known about the initial steps of ovarian differentiation. It is known that Wnt4, as well as the presence of germ cells, are necessary for ovarian differentiation and formation of primordial follicles. Embryonic XX gonocytes differentiate to oogonia and initiate meiotic division up to the stage of diplotene oocytes.

**Internal genital duct differentiation.** In the absence of a functional testis, müllerian ducts differentiate, under Wnt-4 stimulation, to form fallopian ducts, the uterus and the upper third of the vagina. As said before, AMH secreted by pre-Sertoli cells inhibits the müllerian duct by local diffusion between the 9th and 12th week of gestation. This effect is mediated by a specific membrane receptor. On the other hand, fetal Leydig cells secrete testosterone to stimulate Wolf duct differentiation into the vas deference, seminal vesicles and epididymis.

**Differentiation of external genitalia.** In the absence of a fetal functioning testis, or androgens of other sources, the morphology of external genitalia is feminine. Circulating testosterone reaches external genitalia to induce masculinization and prostate formation between the 8th and 13th week of gestation. However, testosterone is a pro-hormone, and it needs to be converted into dihydrotestosterone, by the type 2 5α-reductase enzyme to become active. The mechanism of action requires binding to the specific androgen receptor to stimulate specific genes. The androgen receptor gene is located in chromosome Xq11. It has 8 exons. The corresponding protein has 919 aa with the characteristics of a steroid hormone receptor. It is a ligand-dependent transcription factor binding dihydrotestosterone with greater affinity than testosterone. The dihydrotestosterone-steroid receptor complex is bound to DNA as homodimer to activate transcription in the regulatory region of androgen dependent genes.

**Testicular descent to the scrotum.** Testicular descent to the scrotum during the third trimester of pregnancy completes sexual differentiation in males. This process can be divided in two parts: 1, trans-abdominal migration and 2, inguino-scrotal descent. Trans-abdominal migration is facilitated by regression of the cranial suspensory ligament (induced by testosterone) which connects the testis to the posterior abdominal wall, and by the development of the gubernaculum testis (induced by insulin-like 3, InsL3) which connects the inferior pole of the testis to the scrotum. InsL3, secreted by Leydig cells, has his own receptor, named GREAT (G-protein-coupled-receptor affecting testis descent). The first step brings the testis to the internal inguinal orifice. Finally, inguino-scrotal descent is mediated by retraction of the gubernaculum, stimulated by testosterone.

**Pre-natal or peri-natal brain sex programming.** A long-timed controversy exists regarding the issue of a possible pre-natal androgen effect on brain programming of male sexual identity in adult life. In this regard, sexual conduct and sexual identity can be considered separately [3]. Data in experimental animals and indirect information collected from patients with abnormalities of sexual differentiation suggest that this programming is possible. In our opinion, this possibility is one element to be taken into account, at the time of sex assignment in a newborn with ambiguous genitalia.

**Post-natal activation of the testis (minipuberty).** In the 70s, Forest et al. [4] described that plasma concentration of testosterone, secreted by Leydig cells, peaked during the second or third trimester of post-natal life, to descend later on to very low values. These values remain low from the age of 6 months until the onset of puberty. Lately, it has been described that serum concentration of both inhibin B [5] and AMH [6], products of Sertoli cells, are also high during the first months of life. However, they decrease gradually during the first years of life. The biological effects of this post natal activation
of the testes are poorly understood. However, experimental evidences in monkeys suggest that this activation would be important for adult life. Furthermore, it could participate, along with fetal testis activation, in brain maturation, which is particularly active at this stage of development. In girls, post natal transient activation of gonadotropins has also been described.

**SPECIFIC CLINICAL EXAMINATION**

Clinical interrogation should not omit questioning about the possibility of other family member affected with ambiguous genitalia or similar conditions, precocious puberty or unexplained death during the first months of life (adrenal insufficiency). Moreover, information regarding the existence of maternal family members affected with intersexual conditions, including aunts or grand aunts with amenorrhea (androgen insensitivity syndrome) should be collected. Information on medications received by the mother during pregnancy (particularly because of thread of abortion), or spontaneous virilization of the mother during pregnancy might be important. During the first months of life, information on general wellbeing, feeding difficulties and weight gain might also be of importance.

Careful physical examination should be performed in every case. In the midline, the characteristics of the phallus, its size (length and diameter), degree of curving and frequency of erections should be noted. Documentation of genital and urinary external openings, (frequently only one orifice), their location and diameter and the characteristics of the scrotum or labio-scrotal folds are important. An excessive degree of skin pigmentation might indicate high levels of ACTH stimulation. Finally, a careful search for palpable gonads in labio-scrotal folds and inguinal canals is mandatory. Size, location and palpable characteristics of the gonads should be documented.

**DIAGNOSTIC ORIENTATION**

An approximation to etiological diagnosis is central for decision-making. Due to its frequency and clinical importance, life-threatening salt-losing congenital adrenal hyperplasia (CAH) is the first diagnosis to be considered. These girls frequently have severe dehydration during the first weeks of life. In this context, the weight curve during the first months of life is important to confirm or discard the diagnosis. For these reasons, in all newborns with ambiguous genitalia and non-palpable gonads, an urgent determination of serum 17-hydroxyprogesterone and serum electrolytes is necessary. It has to be taken into account that, on one hand the alteration in serum sodium and serum potassium might take a few days, and on the other, normal values for serum 17-hydroxyprogesterone are higher during the first two weeks of life, particularly in premature babies. Even though to define etiological diagnosis initially might be difficult, it is important to include the patient within one of the groups of the following general classification:

a) 46,XX caryotype with ambiguous or masculine external genitalia and normal ovarian differentiation (female pseudohermaphroditism).

b) 46,XY caryotype with ambiguous or external feminine genitalia and normal testicular differentiation (male pseudohermaphroditism).

c) Gonadal dysgenesis with variable caryotype, frequently abnormal.

In order to arrive to this primary diagnostic approach, it is important to carry out a chromosomal analysis as soon as possible. As an exception, in the case of classical CAH, diagnosis can be made with acceptable degree of certainty with clinical examination and hormonal assays without a caryotype.. These patients belong to group
“a”. Sex can be assigned before confirmation of diagnosis by demonstration of a bi-allelic mutation in the molecular analysis of the CYP21B gene. A second step in diagnosis is to classify the patient in one of the subgroups (secondary diagnostic approximation). This approximation is frequently more complex, and it requires more time and additional studies not always available at the time of sex assignment. A thorough evaluation of this subject can be read in the chapter “Anomalías de la diferenciación sexual” of the book “Tratado de Endocrinología Pediatrica”, edited by M. Pombo [7]). The most important subgroups of patients with ambiguous genitalia are the following:

a) 46,XX caryotype with ambiguous or masculine external genitalia and normal ovarian differentiation (female pseudohermaphroditism).

Internal genitalia (uterus and fallopian tubes) are feminine.
1. CAH secondary to 21-hydroxylase deficiency (CYP21B gene) [8-12]
   - Salt-losing form
   - Simple virilizing form
2. CAH secondary to 11_α-hidroxilase deficiency (CYP11B1 gene) [13]
   Associated to arterial hypertension
3. CAH secondary to 3_α-hydroxysteroid dehydrogenase deficiency (3_α-HSD II gene) [14].
4. Aromatase deficiency (CYP19 gene) [15].
5. Androgenic medication received by the mother during pregnancy
6. Virilizing tumor in the mother during pregnancy
7. Deficiency of the glucocorticoid receptor [16]

b) 46,XY caryotype with ambiguous or feminine external genitalia and normal testicular differentiation (male pseudohermaphroditism).

Müllerian duct derivatives (uterus and fallopian tubes) are inhibited because of adequate secretion and action of fetal AMH secreted by Sertoli cells. Exceptionally, isolated deficiency of AMH or its receptor results in persistence of uterus and fallopian tubes with normal male external genitalia.
1. Deficiency of the androgen receptor (AR). The AR gene is located in the X chromosome [17].
   Also known as testicular feminization syndrome and androgen insensitivity syndrome. Clinical forms:
   - Incomplete: ambiguous external genitalia or micropenis
   - Complete: female external genitalia
   Testosterone secretion is high or normal but there is an absent or incomplete response to androgens.
2. Deficiency of the 5_α-reductase (SRD5A2 gene) [18].
   This enzyme catalyzes the reduction of testosterone to dihydrotestosterone (DHT) in peripheral target organs, such as external genitalia and prostate. DHT is necessary for normal male differentiation of these organs during fetal life. Testosterone secretion is normal but conversion to DHT is deficient.
3. Deficiency of proteins necessary for steroidogenesis (deficiency of testosterone secretion by the fetal testis)
   - Leydig cell hypoplasia (deficiency of LH receptor) [19]
   - Deficiency of 7-dehydrocholesterol reductase (DHCR7 gene)
     Smith-Lemli-Opitz syndrome [20]
   - Deficiency of StAR protein (cholesterol transport through mitochondrial Membranes) [21]
   - Deficiency of 3_α-hydroxyysteroid dehydrogenase (3_α-HSD II gene)*
- Deficiency of 17-hydroxylase (CYP17 gene)* [22]
- Deficiency of 17-hydroxylase/17, 20-desmolase (CYP17 gene)* [23]
  Deficiency of 17_ -_hydroxyesteroid dehydrogenase (17_-HDS III gene) [24, 25]*

Cortisol and aldosterone deficiencies. These two deficiencies are compensated in 17-hydroxylase/17, 20 desmolase deficiency. Arterial hypertension is a prominent sign in complete 17-hydroxylase deficiency.

c) Gonadal dysgenesis with variable caryotype.
This is an important, complex and controversial group of patients, in which major recent advances have been accomplished. If some testis differentiation is present, co-existence of male and female genital ducts is frequently found.

1. True hermaphroditism, i. e., co-existence of testicular and ovarian tissue [26]. This is a heterogeneous cytogenetic condition. Chromosomal constitution can be:
   a) 46,XX (66 %). In a few cases, translocation of the SRY gene to an autosome or to chromosome X has been shown [27]. In other cases, it has been postulated that deficiencies in autosomal genes active in testicular differentiation could be present [28, 29].
   b) 46,XX/46,XY (27 %) or other cellular mosaicsms [30].
   c) 46,XY (7 %). A loss of function mutation of the SRY gene [31] or a dissociation between peripheral blood (46,XY) and gonad caryotype (46,XX/46,XY)

2. Mixed gonadal dysgenesis or asymmetric gonadal differentiation [32].
There is differentiation of a testis on one side, and an undifferentiated gonadal streak on the other. Caryotype is usually 46,XY/45,X0, but other variants can be present. Congenital anomalies similar to those found in Turner syndrome are frequent. It has been postulated that the double dose of DAX-1 located in the X chromosomes, even though they are in different cell lines, might be the explanation for the regression of one of the gonads [2].

3. Testicular dysgenesis with 46,XY caryotype [2].
This condition is also named dysgenetic male pseudohermaphroditism (or XY sex reversal in extreme cases). Variable degrees of testicular differentiation, and therefore of the two functions of the testis, might occur. In extreme cases, external genitalia are female (Swyer syndrome), but partial deficiencies with ambiguous genitalia can occur. In the last decades, important advances in the understanding of testicular differentiation have been made (see Figure 1). Testis differentiation is triggered by SRY acting on the undifferentiated bisexual embryonic gonad. Inactivating mutations of SRY gene have been found in a few patients [33]. Loss-of-function mutations in some autosomal genes active in testicular differentiation or excessive dose of inhibiting autosomal or X chromosome genes (such as DAX-1) can also be responsible of lack of adequate differentiation [34]. Some of the inactivating mutations are part of specific syndromes, such as Frasier and Denys-Drash syndromes, which associate gonadal and renal abnormalities secondary to loss of function mutation of the transcription factor WT1 gene [2]. Furthermore, deficiency of SF-1, a factor which regulates the expression of multiple genes necessary for sexual differentiation, has been found in dysgenetic male pseudohermaphroditism [35]. Campomelic dysplasia, a syndrome associating testicular dysgenesis
and skeletal anomalies, is secondary to a loss of function mutation of the transcription factor SOX9 gene [36]. Alternatively, an excessive dose of testicular differentiation inhibiting genes can produce testicular dysgenesis. As mentioned earlier, a double dose of DAX-1 (dose sensitive sex reversal locus) inhibits fetal testis differentiation [37]. Moreover, an excessive dose of the signaling molecule WNT-4 has been detected in some patients with dysgenetic male pseudohermaphroditism. Over expression of WNT-4 results in an increase of DAX-1, which in turn inhibits testicular differentiation [38]. However, it has to be stressed that, in most cases of 46,XY testicular dysgenesis, etiology remains unknown.

4. Pure gonadal dysgenesis (Swyer syndrome). Either 46,XY or 46,XX caryotype caryotype can be found in these patients. Since gonads do not differentiate, external and internal genitalia are feminine.

5. 46,XX male or testicular dysgenesis with 46,XX chromosomal constitution. Multiple mechanisms are possible. It can be secondary to a dose excess (gene duplication) of genes active in testicular differentiation, or to a translocation of SRY into an X chromosome. A dissociation between the genetic material in circulating lymphocytes and in gonadal cells has also been reported [39]. Usually, genitalia are not ambiguous except for mild hypospadias. However, in patients with excessive dose of SOX9, genitalia are ambiguous [40].

6. Klinfelter syndrome, 47,XXY or variants [41]. External genitalia are not ambiguous.

7. Turner syndrome, 45,X or variants [42]. Gonadal atresia with female external genitalia.

d) Genital malformations associated with multiple congenital malformations

This heterogeneous group raises difficulties in sex assignment, particularly when intellectual functions are normal.

An example is cloacal extrophy with bladder extrophy [43]. It has been published that a large proportion of 46,XY subjects assigned to the female sex are not satisfied or have changed sex in adult life [44].

SOCIAL AND LEGAL SEX ASSIGNMENT

As we have already analysed, multiple abnormalities can result in a newborn with ambiguous genitalia. Even though in many occasions sex assignment has to be decided without a precise diagnosis, it is important to have a diagnostic approximation for a better decision. The basic aim is to assign the sex which should the best option for the newborn future life. This aim is, at best, only a good intention. Often, decisions are difficult and controversial, and, frequently, no good solution is available. We are not in favour of postponing sex assignment until late adolescence. In a newborn with ambiguous genitalia, the potential functional capacity of external and internal genitalia, as well as the possibilities of surgical corrections, should be taken into account. Important goals are future self-identification with assigned sex, compatible sex behaviour and familial and social acceptance as such. At this point, post-natal experiences, so important later in life, are not present yet. However, there are direct evidences in experimental animals (and indirect evidences in humans) that pre-natal hormones (androgens) may act on fetal brain programming sex conduct in future life. In
the second part of this review, we will analyze the process of decision-making in different patient sub-groups.

Classical basic concepts regarding sex assignment in newborns have been questioned in public by some patients [44], but a recent poll has shown that most adult 46,XY intersex patients are satisfied with the assigned sex and with their genitalia. A significant minority, however, is not satisfied and proposed a change [45].

SECOND PART ANNOUNCEMENT

In the second number of Endocrinología Pediátrica On line we will continue with the second part of the Review THE NEWBORN WITH AMBIGUOUS GENITALIA. NEW DIAGNOSTIC CONCEPTS. CLINICAL SCENERIES AND DECISIONS. In this part, we will describe the most frequent clinical sceneries for the medical team, in an effort to provide some help for the treating physicians. These clinical sceneries are based on the clinical examination of the newborn and on the characteristics of the caryotype.

REFERENCES

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