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THERAPIES

HEADING: Letter to editor

Letter to the Editor concerning the high incidence of MRKH syndrome in third-generation DES women

Daniel Guerrier^{a*}, Sylvie Epelboin^b, Michel Tournaire^c

^a *Univ Rennes, CNRS, INSERM, IGDR (Institut de Génétique et Développement de Rennes), UMR 6290, ERL U.1305, 35000 Rennes, France*

^b *Assistance publique – Hôpitaux de Paris-Nord, Université de Paris, Hôpital Bichat-Claude Bernard, Service de Gynécologie-Obstétrique, 75018 Paris, France*

^c *Université de Paris, 75006 Paris, France*

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***Corresponding author.** Univ Rennes, CNRS, INSERM, IGDR (Institut de Génétique et Développement de Rennes), UMR 6290, ERL U.1305, 35000 Rennes, France.

E-mail address: daniel.guerrier@univ-rennes1.fr (D. Guerrier)

KEYWORDS

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Abbreviations

AMH: anti-Müllerian hormone

DES: diethylstilbestrol

MDs: Müllerian (paramesonephric) ducts

MRKHS: Mayer-Rokitansky-Küster-Hauser syndrome

PGCs: primordial germ cells

UGT: upper genital tract

We would like to provide additional genetic information to the questions raised by Wautier *et al.* [1]. In their retrospective study, the authors evaluated the incidence of genital malformations, fertility, and pregnancy outcomes in third-generation women whose mothers or fathers were prenatally exposed to diethylstilbestrol (DES). Among the 12 cases of uterine malformations reported (1.6% of the cohort), there were three cases of congenital utero-vaginal aplasia, a defining characteristic of Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS) [2]. These three cases represent an incidence of 1 in 250 of the total cohort, which is 20 times the prevalence in the general population, suggesting a link between DES exposure and MRKHS. A previously unpublished familial situation reinforces this hypothesis; among three sisters exposed *in utero* to DES, two had MRKHS and the third had uterine malformations typical of fetal DES exposure. In view of this situation, the following questions arise: is there a causal link between DES exposure and MRKHS for (1) the second-generation “DES daughters” exposed *in utero* and (2) the third-generation “DES granddaughters”?

A review of the development of the human reproductive system

The development of the female upper genital tract (UGT) follows two distinct phases. The early phase, known as the initiation of the Müllerian (paramesonephric) ducts (MDs), takes place during

weeks 8 and 9 of pregnancy (from the first day of the last menstrual period) in the undifferentiated XX or XY embryo. MDs originate at the apical part of the mesonephros (primitive kidney) and elongate caudally along Wolff's excretory (mesonephric) ducts [3]. From a genetic point of view, the initiation of MDs does not depend on chromosomal sex and involves genes independent of sex steroids, such as *PAX2*, *LHX1*, *TCF2*, *DMRT1*, and *WNT-4*, *-7A*, and *-9B*, and those encoding retinoic acid α - and β -receptors [3] (Fig. 1).

During the second phase, from the 10th week onwards, in XX individuals and in the absence of anti-Müllerian hormone (AMH), MDs persist and undergo a second (anatomical) step of differentiation: the apical section gives rise to the Fallopian tubes, while the medial and caudal parts fuse to form the uterus and the upper part of the vagina. The anteroposterior patterning into tubes, uterus, cervix, and vagina is under the control of segmental activation of the *HOXA-9*, *-10*, *-11*, and *-13* genes, respectively [4]. The entire UGT is thus formed morphologically by the 22nd week. There is no evidence that the anatomical differentiation of MDs depends on estrogens [3]. However, it is established that *HOXA-10* and *-11* are able to respond to estrogens, which otherwise control their expression in the adult endometrium [4].

In parallel to Müllerian differentiation, primordial germ cells (PGCs), which are at the origin of third-generation individuals, proliferate from the neural crest and invade the undifferentiated fetal gonad between weeks 6 and 12 [5]. During this process, PGCs undergo epigenetic deprogramming through progressive demethylation. The parental imprints are erased and the inactive X chromosome is reactivated [5]. In females, meiosis (oogenesis) takes place from weeks 12 to 22, but is rapidly blocked in the prophase of meiosis I, conferring a quiescent state to the oogonia (future oocytes). The oogonia are then progressively surrounded by granulosa cells to form primordial and then primary follicles, and undergo reprogramming from week 30 until birth. Completion of meiosis is induced by fertilization. In males, reprogramming takes place between weeks 12 and 30, during which time the mitotic proliferation of the pro-spermatogonia gradually stops. Meiotic divisions (spermatogenesis) only start at puberty [5]. The period of DES intake, from the 7th week until four weeks before term [6], covers the period of epigenetic reprogramming of both female and male gametes which are then vulnerable to exogenous agents such as DES, capable of disrupting the regulation of methylation enzymes [7].

Abnormal development of the female upper genital tract: MRKH syndrome and the effects of DES exposure

Current knowledge and genetic considerations on MRKH syndrome (MRKHS)

MRKHS is the second most common cause of primary amenorrhea after gonadal dysgenesis and affects approximately 1 in 4,500 women [2]. It is characterized by congenital aplasia of the uterus, the upper two-thirds of the vagina, and sometimes the Fallopian tubes in XX women with otherwise normal sexual characteristics and hormonal status. Uterovaginal aplasia can be either isolated (Type 1 MRKHS) or associated with other malformations (Type 2 MRKHS) that can affect the kidney, spine and, to a lesser extent, the otological or cardiac sphere [2].

In embryogenic terms, MRKHS can be considered either as a defect in the elongation of the MDs, when only tubal residues are present, or as a defect in the onset of anatomical differentiation, resulting in a hypoplastic or entirely absent tract. From a genetic point of view, anomalies of non-steroid-dependent genes involved in the early phase of UGT development must be evoked. This is all the more likely, as associated malformations in type 2 MRKHS are attributable to anomalies of genes involved in more general early embryonic insults. As an example, the *GREBIL* gene, encoding a cofactor of the retinoic acid receptor, is necessary for the correct establishment of the mesonephros. *GREBIL* has recently been implicated in several familial or sporadic cases of Type 2 MRKHS, in which associated malformations were found to most often be renal and more rarely skeletal [8]. Other *GREBIL* variants have also been identified in congenital deafness [8], which is optionally associated with Type 2 MRKHS [2]. This adds *GREBIL* to the list of pleiotropic non-estrogen-dependent genes necessary for proper development of the UGT. On the other hand, the *HOXA-9* to *-13* genes, involved in the anatomical differentiation of the UGT (late phase), have, thus far, not been implicated in MRKHS.

Effects of DES exposure in second- and third-generation women

Second-generation women prenatally exposed to DES show specific clinical signs: menstrual irregularity, infertility, and various pregnancy complications (miscarriage and premature birth,

ectopic pregnancy), as well as an abnormally high rate of cervico-vaginal clear-cell adenocarcinoma [1]. The functional defects observed appear to be attributable to the reproductive tract malformations characteristic of fetal DES exposure, namely a T-shaped, hypoplastic uterus with irregular edges and sub-cornual constriction. This abnormal structure of the uterus, together with tubal anomalies and vaginal adenosis (presence of glandular tissue typical of the endocervix and endometrium), suggests a severe alteration of the HOX program, leading to a posterior shift in the acquisition of identity of each segment of the UGT, similar to that observed with *HOXA-10* or *HOXA-11* gene inactivation in mouse models [4]. Indeed, both the *HOXA-10* and *-11* genes undergo a double blockage: first, a direct antagonistic action of DES mediated by the α -estrogen receptor [7] and, second, hypermethylation following prolonged DES exposure [7]. Finally, the frequency of malformations in second-generation women has been reported to depend on the starting date of fetal exposure: 73% before the 12th week, 66% from weeks 13 to 18, and 45% after the 18th week [9]. This observation appears to corroborate the hypothesis of deregulation of the HOX program, which takes place in the fetus from the 10th week: the earlier disruption, the greater the frequency and extent of malformations.

Third-generation women, whose parents were exposed *in utero* to DES, also show uterine malformations suggestive of a transgenerational effect of DES exposure [1]. This effect can only be explained by direct epigenetic modifications induced by DES that affect the genome of the gametes (oocytes or spermatozoa) of second-generation parents (see section 1).

Analysis of situations

Second-generation DES daughters with MRKH

In the aforementioned family, three daughters were exposed *in utero* to DES. The two first non-twin daughters showed Type 1 MRKH syndrome, with absence of the vagina, a rudimentary uterus, normal Fallopian tubes, and no other associated malformations. For these two sisters, DES exposure occurred early in pregnancy due to a maternal history of miscarriages. For the third sister, DES intake occurred later, at the end of the first trimester. The uterus was described as “slightly T-shaped”, and she gave birth to three children.

MRKH syndrome is an early dysfunction of MD differentiation and is attributable to anomalies of non-estrogen-responsive genes (see above). In contrast, the direct effects of DES in the second generation take place later, after the onset of anatomical differentiation of the uterus (Fig. 1). This appears to exclude the involvement of DES in MRKHS from a chronological perspective. The epidemiological analysis of uterine malformations provides another convincing argument against a role for DES in MRKHS; millions of women worldwide have been exposed to DES *in utero* and MRKHS is not one of the adverse effects reported to date. Conversely, the familial or sporadic cases of MRKHS reported in the literature were never linked to DES exposure, whereas the number of genetic diagnoses is steadily increasing.

In the third sister, the presence of uterine malformations characteristic of DES exposure attests to the correct initiation of Müllerian differentiation. The malformations described as "slight" are related to late exposure to DES and therefore to late disruption of the HOX program. The direct relationship between the starting date of DES intake and both the frequency and, probably, the severity of adverse effects on the fetus [9] reinforces this hypothesis. In other words, these observations support the separate timing of uterovaginal aplasia and the effects of DES, this chemical affecting the anatomical differentiation of a previously established embryonic uterus. Finally, a gene anomaly is likely responsible for the MRKHS of the two older sisters, and it appears that the third daughter did not inherit the morbid allele from one of her parents.

Third-generation (granddaughters) DES

Wautier et *al.* investigated genital malformations in 759 third-generation DES granddaughters and reported three cases of MRKHS. Two were from among 672 "DES daughters" and one was from among 76 "DES sons" [1]. These rates are surprising, as they are much higher (~20X) than expected in the general population [2].

This study revealed that genital anomalies found in third-generation DES daughters (doubling, bicornuate uteri, and MRKHS) are different from those of the second generation (T-shaped uterus) and identical to those observed in the general population and about as frequent. Concerning the frequency of MRKHS in the cohort of interest, we cannot a priori exclude a causative epigenetic modification of a gene, in particular, for Type 1 MRKHS, in which only the UGT is affected. However, this is the first reporting of MRKHS among the numerous third-generation cohorts already

studied, raising the question of the significance of its apparent frequency. On the contrary, a significantly high frequency of hypospadias has been found in several studies of third-generation men [10]. This evokes an epigenetic alteration of a unique causative gene, the strongest hypothesis being hypermethylation of the chromosomal locus comprising the genes of the *HOXA* family, in particular, the *HOXA-13* gene. Indeed, *HOXA-13* is known to be involved in both hypospadias and incomplete Müllerian fusion [11], both observed in the third DES generation, which reinforces this assumption.

Conclusion

The hypothesis of a causal relationship between DES exposure and MRKH syndrome appears to be unlikely. Among second-generation women, the time lag between the initial non-estrogen-dependent formation of Müllerian ducts and their anatomical differentiation controlled by estrogen-sensitive *HOX* genes rules out this hypothesis. For third-generation women, epigenetic alteration of an MRKHS gene is rather unlikely, as MRKH cases would then have been numerous and recurrent among the millions of women concerned. However, the incidence of genital malformations among third-generation men strongly supports a transgenerational effect of DES. Efforts must be pursued to study the largest possible cohorts with the aim of definitively establishing the impact of DES on third-generation women and men.

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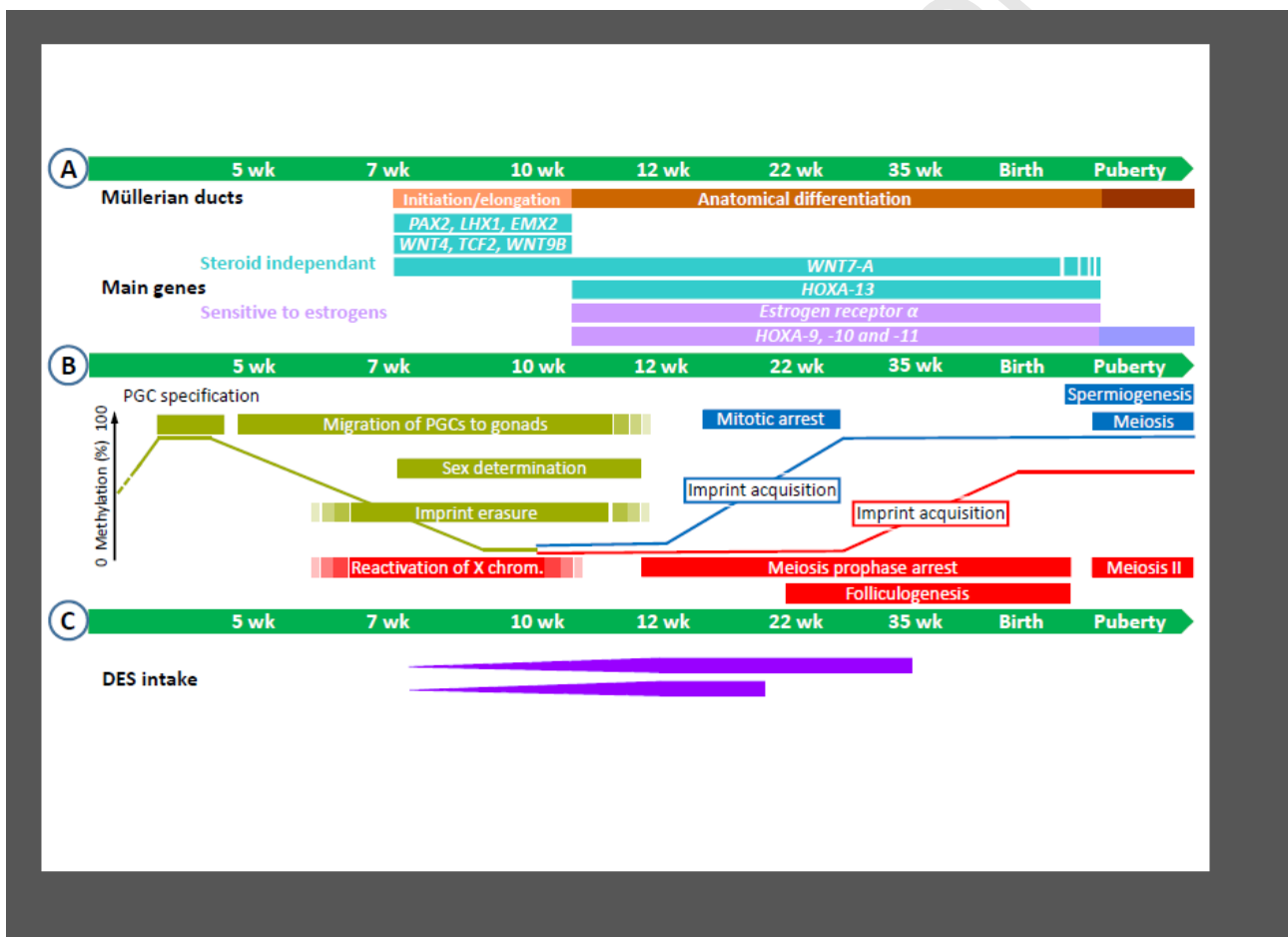
Disclosure of interest

The authors have no conflicts of interest to declare.

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Figure 1. Establishment of human reproductive functions. (A) Müllerian duct formation and differentiation and key gene expression. (B) Gametogenesis and epigenetic changes. (PGC: primordial germ cells). Sexually undifferentiated period: olive green, post sexual differentiation: male - blue, female - red. (C) DES intake during pregnancy (starting between the 7th and 15th week and ending around the 22nd to 35th week). The green bars indicate the time of pregnancy (non-linearly) in weeks from the first day of the last menstrual period (A, B, and C).



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