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IDENTIFICATION OF GENETIC FACTORS INVOLVED IN CONGENITAL UTERINE ANOMALIES

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« Je ne vois qu'un moyen de savoir jusqu'où on peut aller.
C'est de se mettre en route et de marcher. »
Henri Bergson

Abstract

Ranging from aplastic uterus (including Mayer–Rokitansky–Küster–Hauser syndrome) to incomplete septate uterus, uterine malformations as a group are relatively frequent in the general population. Specific causes remain largely unknown. Recurrent copy number variants are found in up to 14% of girls with Müllerian aplasia, and only a few genes have garnered strong evidence of causality, mainly in syndromic presentations. Although most occurrences ostensibly seem sporadic, familial recurrences have been observed, strongly indicating genetic factors. The aim of this thesis was to identify novel genes and pathways involved in congenital uterine anomalies. To increase the chance to identify strong genetic contributors, we selected families with recurrence of uterine and kidney malformations for genetic analyses by whole exome sequencing. Nine families were collected throughout the study. Exome sequencing analyses uncovered likely causative variations in the gene *GREB1L* for four of these families. In a fifth family, the candidate gene *NR6A1* was selected for further functional validation. Its role in renal development and development of the genital tract was investigated in zebrafish. Both genes were sequenced in a cohort of individuals with MRKH syndrome, identifying additional heterozygous variants. Following these results, we discuss some pathways possibly involved in congenital uterine anomalies.

Résumé

Depuis l'absence d'utérus (incluant le syndrome de Mayer–Rokitansky–Küster–Hauser) jusqu'à la présence d'un utérus partiellement septé, les malformations utérines varient dans leur sévérité et sont, dans leur ensemble, relativement fréquentes dans la population générale. Leur étiologie reste largement inconnue. Des variations chromosomiques récurrentes sont identifiés chez moins de 14% des filles avec aplasie müllerienne, et seuls quelques gènes ont prouvé leur causalité, principalement dans des présentations syndromiques associant malformations utérines et d'autres symptômes. Alors que les malformations utérines surviennent de manière sporadique dans la plupart des cas, des présentations familiales ont été décrites, suggérant l'implication de facteurs génétiques. L'objectif de cette thèse est d'identifier de nouveaux gènes et voies moléculaires impliqués dans les anomalies congénitales de l'utérus. Afin d'accroitre nos chances d'identifier des contributeurs génétiques, nous avons sélectionné des familles avec récurrence de malformations utérines et rénales chez plusieurs apparentés. Au total, neuf familles ont ainsi pu être analysées par séquençage d'exome durant cette étude. Des variations probablement causales ont été identifiées dans le gène *GREB1L* pour quatre de ces familles. Dans une cinquième famille, un nouveau gène candidat, *NR6A1*, a été identifié et étudié chez le poisson zèbre afin d'investiguer son rôle potentiel dans le développement du système rénal et du tractus génital. *GREB1L* et *NR6A1* ont de plus été séquencés dans une cohorte d'individus avec syndrome MRKH, permettant d'identifier plusieurs variants hétérozygotes additionnels dans ces gènes. Suite à ces résultats, nous discutons certaines voies moléculaires impliquées dans les anomalies congénitales de l'utérus.

Remerciements

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Abbreviation

AARR	Al-Awadi/Raas-Rothschild
ACMG/AMP	American College of Medical Genetics and Genomics (ACMG), Association for Molecular Pathology (AMP)
AIM	anterior intermediate mesoderm
AMH	anti-Müllerian hormone
AR	androgen receptors
ATRA	all-trans retinoic acid
BMP	bone morphogenic protein
CAKUT	congenital anomalies of the kidney and urinary tract
CBABD	congenital bilateral absence of the vas deferens
CM	cap mesenchyme
CNV	copy number variation
CRISPR	clustered regularly interspaced short palindromic repeats
CUA	congenital uterine anomalies
DE	distal early
DEAB	diethylaminobenzaldehyde
DES	diethylstilbestrol
DL	distal late
DNA	deoxyribonucleic acid
EDC	endocrine-disrupting chemical
EMSA	electrophoretic mobility shift assays
ENU	N-ethyl-N-nitrosourea
ESHRE/ESGE	European Society of Human Reproduction and Embryology/European Society for Gynaecological Endoscopy
FGF	fibroblast growth factor
FGT	female genital tract
FSH	follicle-stimulating hormone
G	glomerule
HFG	hand–foot–genital
Hpf	hours post-fertilization
LH	luteinizing hormone
MDA	Müllerian duct anomalies
MODY	maturity onset diabetes of the young
MRI	magnetic resonance imaging
MRKH	Mayer-Rokitansky-Kuster-Hauser syndrome
MURCS	Müllerian, renal, and cervicothoracic somite

MD	Müllerian ducts
N	neck segment
NGS	next generation sequencing
NHEJ	non-homologous end joining
NMP	neuromesodermal progenitors
OHVIRA	obstructed hemivagina, and ipsilateral renal agenesis
OMIM	online mendelian inheritance of man database
PAM	protospacer adjacent motif
PCT	proximal convoluted tubule
PD	pronephric duct
PIM	posterior intermediate mesoderm
PST	proximal straight tubule
PTH	parathormone
RA	retinoic acid
RCAD	renal cysts and diabetes syndrome
RME	random autosomal monoallelic gene expression
RNA	ribonucleic acid
SCD	spondylocostal dysostosis
smMIP	single-molecule molecular inversion probes
SNP	single nucleotide polymorphism
TAR	thrombocytopenia-absent radius) syndrome
TGO	glutamate-oxaloacetate-transaminase
TGP	glutamate-pyruvate-transaminase
TSH	thyroid Stimulating Hormone
UB	ureteric bud
UGS	urogenital sinus
UTx	uterine allotransplantation
UVC	uterovaginal canal
VACTERL	acronym for vertebral defects (V), anorectal malformation (A), cardiac anomalies (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R) and limb malformation (L)
WD	Wolffian ducts
WES	whole exome sequencing
WISH	whole-mount is situ hybridization
WNT	abbreviation for wingless, the Drosophila segment polarity gene, and integrated (int-1), the vertebrate analog.
ZFIN	The Zebrafish Information Network

INTRODUCTION

INTRODUCTION

In the introduction, we will begin by exploring the development of the uterus during embryonic and fetal stages, highlighting the close spatiotemporal relationship between the renal and uterine systems. Following this, we will present the current understanding of congenital uterine anomalies, including their classification, prevalence, and commonly associated malformations. We will also discuss their recurrence risk, along with the genetic and environmental factors identified to date. Some of this information was previously published in a review paper in 2016 (Etiologies of Uterine Malformations, American Journal of Medical Genetics, 2016- enclosed in the Appendix). Lastly, we will introduce the criteria used for interpreting exome data and emphasize the utility of the zebrafish model in studying urogenital developmental anomalies.

I. EMBRYOLOGICAL ORIGIN OF THE UTERUS

The uterus, with the fallopian tubes, cervix and vagina, constitute the internal ductal system of the female reproductive organs, which also include the gonads (ovaries in females) and the external genitalia. The embryonic anlagen of the uterus and of most of the female reproductive tract are the paramesonephric or Müllerian ducts which appear during the first trimester in the embryo, and then differentiate, mature and grow during the fetal and postnatal life (1).

In this section, we will first review the different steps leading to the formation of the female reproductive tract during the embryonic (up to the 9th week after conception) and fetal life in humans. We will then discuss the current knowledge in regards to the main pathways and transcription factors known to be involved in the development of the uterus based on animal models.

Finally, the development of the renal and urinary system will also be explained briefly given the frequent association of renal and urological malformations in patients with Müllerian ducts anomalies, and the interaction between the mesonephric and paramesonephric ducts during their development (2).

1. DEVELOPMENTAL STEPS OF THE FEMALE REPRODUCTIVE TRACT IN HUMANS

At the 5th week post-ovulation, the mesonephros (Wolffian ducts) have reached the cloaca (Figure 1.A) and formation of the Müllerian ducts begins in both male and female human embryos with invagination from a group of precursor cells in the coelomic epithelium within the intermediate mesoderm (BOX1)[1] at the level of the 3rd thoracic somite. The Müllerian ducts then elongate in an anterior to posterior direction to reach the urogenital sinus around the 8th week. The point of contact is named the Müllerian tubercle or sinusal tubercle (1). During their elongation to the urogenital sinus, the Müllerian ducts are located in the dorsolateral edge of the mesonephric kidneys, in close proximity with the Wolffian ducts, especially in their most caudal part where epithelium of both Wolffian and Müllerian ducts are in direct contact (Figure 1.B).

At the bipotential stage (6 weeks), both Müllerian and Wolffian ducts are present in male and female embryos within the urogenital ridges (Figure 1.C). Following gonadal differentiation (ovaries in XX female and testes in XY male), the epithelium of the Wolffian ducts involute in female in the absence of testis-derived androgen and the Müllerian ducts regress in male (at about 8.5w) due to the presence of the Anti-Müllerian Hormone (AMH) produced by the testes.

During the 8th week, in females, the Müllerian ducts fuse in a caudal to cranial progression, their lumen being separated by a midline epithelial septum. Secondary to regression of this septum at the 9th week, one cavity (called the uterovaginal canal) will be formed, lined throughout with pseudostratified columnar Müllerian epithelium (Figure1.D)(1).

From the 9th week onward, the uterovaginal canal will differentiate into the Fallopian tubes (or uterine tubes), the uterus corpus, the uterine cervix and the vagina. The cranial unfused portions of the Müllerian ducts give the Fallopian tubes and their extremities (the fimbriae) open into the abdominal cavity. The uterus corpus differentiates from the cranial part of the uterovaginal canal and adopts, from the 9-10 weeks onward, a delta-shape resulting from a medial-lateral expansion that is more significant in the cranial part. The uterus corpus is lined by a columnar epithelium, with glands appearing at about 14-15 weeks. The boundary between the body of the uterus, and the cervix (developing from the

[1] Gastrulation and formation of the intermediate mesoderm is briefly discussed at the end of the section in BOX1.

middle of the uterovaginal canal) is not yet discernable based on their epithelium and glands (Figure 1.E). Similarly, the boundary between the cervix and vagina is unclear until the 18-20 weeks when the vaginal fornix can be visualized. In adult nulliparous women, a columnar mucinous epithelium and stratified squamous epithelium characterize respectively the supravaginal part (endocervix) and the vaginal part (exocervix) of the cervix.

Finally, at 12 weeks, a solid (occluded) vaginal plate forms following the contact between the urogenital canal and the urogenital sinus and upgrowth of the urogenital sinus epithelium. The vaginal cavity then starts to canalize at 16 weeks and is nearly complete at 19 weeks. The relative contribution of the Müllerian ducts versus the urogenital sinus to the squamous epithelium of the human vagina has been conflicting in the literature, but recent observations support its origin solely from the urogenital sinus (1). Myometrial development starts at the level of the cervix at the 11-12 week and is complete for all the female reproductive tract by 20 weeks (1,3). The entire female genital tract is morphologically formed by week 22 (4).

The developmental steps of the uterus development are detailed in Figure 2.

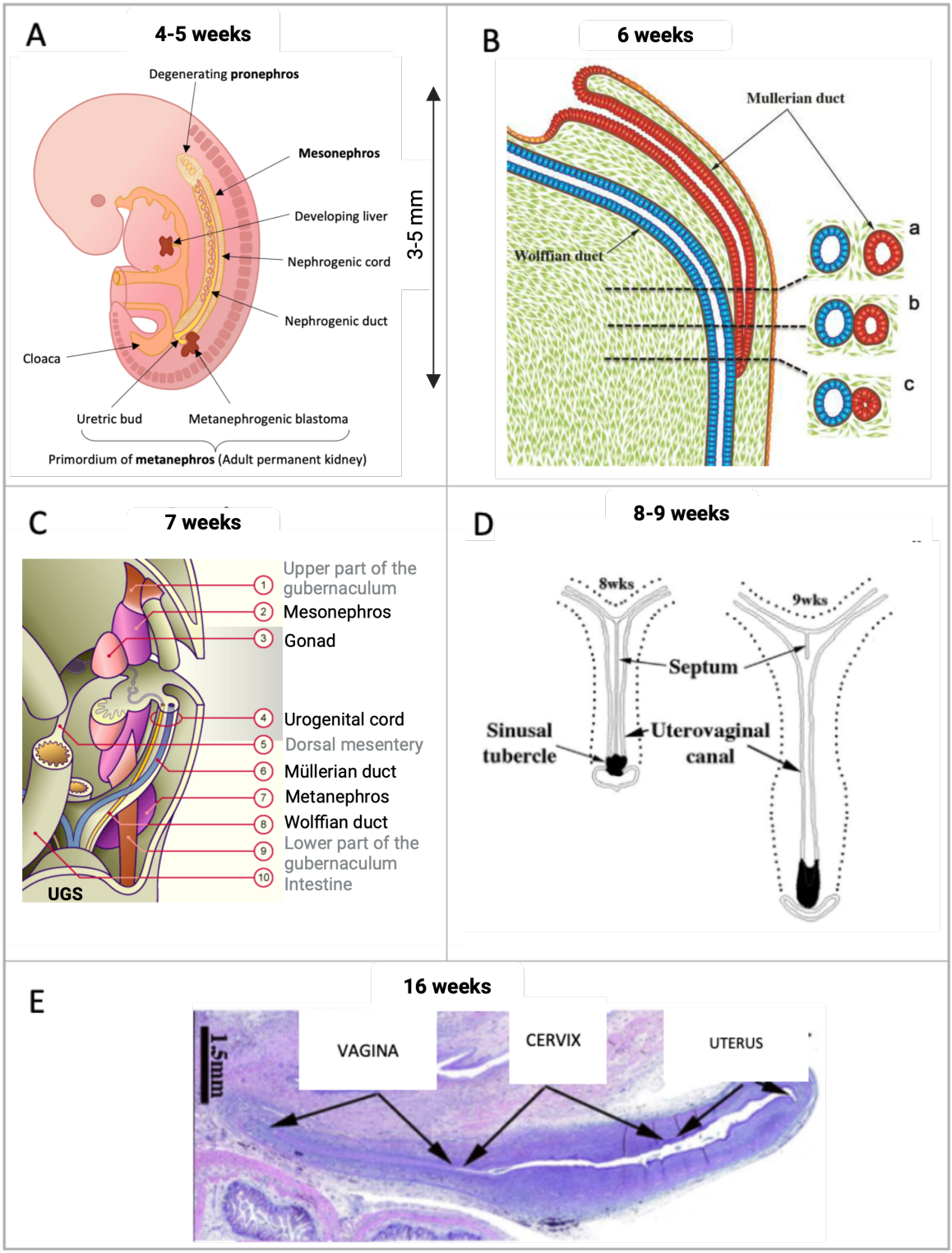


Figure 1. Early stages of urogenital development
Figure A from Kakun 2022 (5); figures B-D-E from Robboy et al 2017 (1); figure C from (6).
UGS= urogenital sinus.

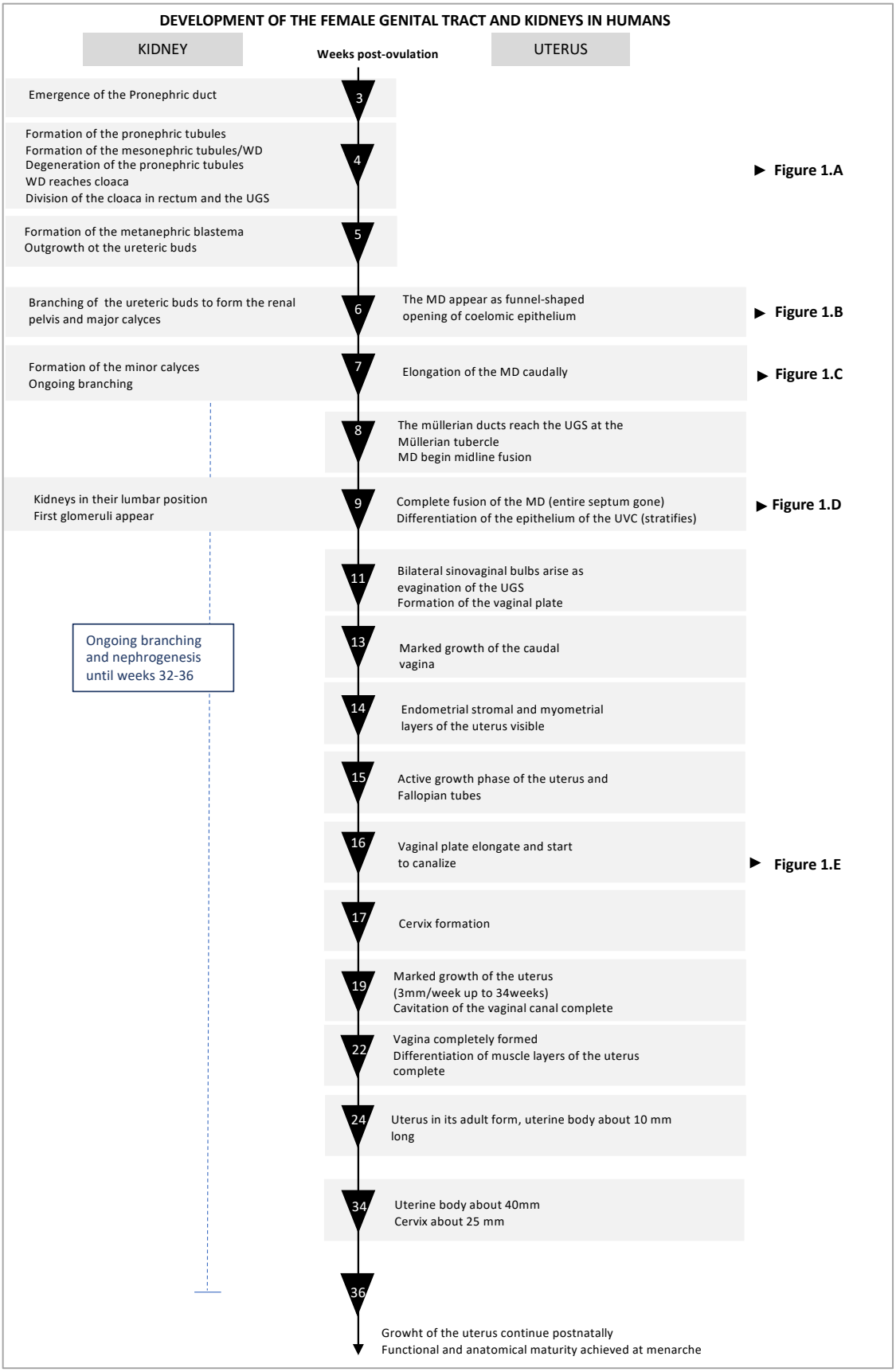


Figure 2. Developmental steps of the female genital tract and kidneys during embryonic and fetal development.
MD= Müllerian ducts; WD=Wolffian ducts; UVC= uterovaginal canal; UGS=urogenital sinus. Adapted from Robboy 2017 (1).

2. GENES AND PATHWAYS INVOLVED IN MÜLLERIAN DUCT DEVELOPMENT

Animal studies (mainly in mouse and chicken) have been useful in improving our understanding of the signaling pathways and transcription factors involved in Müllerian ducts development. Although differences exist in the morphology of the genital tract between mice and human, remarkable similarities in the molecular pathways involved in the formation of the Müllerian ducts were demonstrated (especially for the genes involved in the first steps of duct formation) (7–10). A high degree of conservation does also exist for other vertebrates (e.g., avian). Targeted mutagenesis, mainly in mouse, and gene expression analyses allowed to identify several genes essential for the female reproductive tract development.

Formation and differentiation of the Müllerian ducts

Formation and differentiation of the Müllerian ducts can be divided in four phases (Phases A to D below) in order to describe chronologically the molecular mechanisms involved (illustrated in Figure 3 and summarized below based on Santana Gonzalez et al. [2021] (8), Roly et al. [2018] (11) and Major et al. [2022] (10)). The genes known to be important for Müllerian development, based on studies in animal models, are detailed in Table 1 and will be reviewed in Section III.2.

PHASE A: PROGENITOR'S SPECIFICATION AND PLACODE FORMATION

Müllerian cell progenitors proliferate at the cranial mesonephric coelomic epithelial surface to form a multilayered epithelial thickening (named "a placode"). They go through a process of partial or total epithelial-mesenchymal transition to assume mesoepithelial[2] or mesenchymal phenotypes. In chicken, the placode development depends on BMP signaling, and the FGF pathway was shown to be important for the rupture of the basal membrane, one of the first observable steps of epithelial-mesenchymal transition.

The progenitor cells progressively express different transcription factors. Hence, specification of epithelial coelomic cells into mesoepithealial progenitors is characterized by sequential upregulation of the transcription factors PAX2, EMX2, and LIM1 (LHX1) (and also PAX8, PBX1, HNF1B, WNT7A, DACH1/2 in mouse), while expression of WNT4 (and DMRT1 in chicken) marks the differentiation into mesenchymal progenitors. The

[2] The Müllerian duct epithelium is mesoepithelial in nature, sharing characteristic of mesenchymal cells arranged on a basement membrane (typical of an epithelia).

Müllerian duct epithelium derives exclusively from mesoepithelial progenitors whereas the surrounding mesenchyme include mesenchymal progenitors from the placode as well as mesenchymal cells migrating from the coelomic mesonephric epithelium (8).

PHASE B: INVAGINATION

LIM1 (expressed by mesoepithelial progenitors) and WNT4 (expressed by mesenchymal progenitors) controls the process of invagination. Cell intercalation-mediated apical constriction is the physical mechanism responsible for the invagination (a mechanism depending on an actomyosin skeleton and FGF signaling). The PI3K/AKT pathway is also activated at this stage in rodents (unknown role). The tips of the Müllerian ducts reach the Wolffian ducts.

PHASE C: CAUDAL ELONGATION

The Müllerian ducts progressively elongate caudally though apical constriction, cells proliferation and migration. At the same time, the proliferation of cells expands the tubes in the dorso-ventral axis. The tip of the Müllerian ducts stays in close contact with the epithelium of the Wolffian ducts while progressive proliferation of mesenchymal cells accumulates between the basal membranes of both ducts in more cranial location. The Wolffian ducts are essential during development for Müllerian duct elongation. Besides serving as physical guides, paracrine secretion of WNT9B and GATA3 by the Wolffian ducts are necessary for elongation. Other not yet identified factors could be involved, as well as graded morphogens acting as attractive factors (caudally) or repulsive factors (cranially) to promote the elongation. The GPR56 receptor (expressed by the mesoepithelial cells), WNT4 and DMRT1 (expressed by the mesenchymal cells), the PI3K/AKT pathway, other WNT molecules downstream of WNT4 (WNT5A, WNT7A) and paracrine signals from the coelomic epithelium were shown to play a role in elongation in mice and/or chicken studies (8,11,12). RA signaling is required for Müllerian duct formation and/or elongation although its exact role remain unclear (11).

Fusion of the Müllerian tips with the endoderm-derived urogenital sinus is prevented in *Lhfpl2* knockout mice, showing a role for this transmembrane protein of unknown function (13). Retinoic acid signaling, TGF-beta and Wnt pathways may also play a role in this process (14).

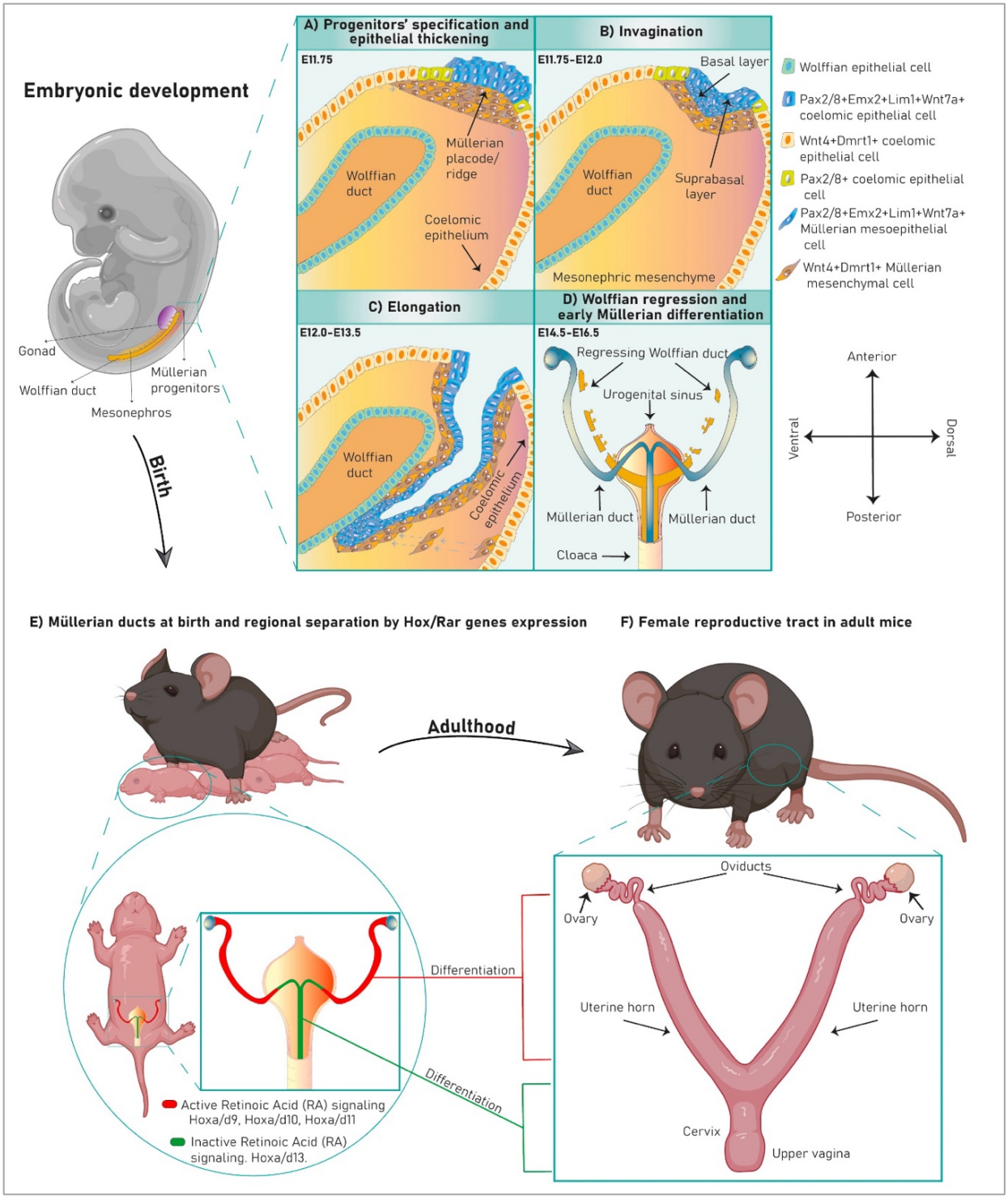


Figure 3. Phases of Müllerian ducts formation and differentiation.
Formation (A-D) and differentiation (E) in mouse and the main transcription factors and pathways involved. The morphological differences with the human female genital tract mainly result from differences in the extent of fusion of the two Müllerian ducts anteriorly. Based on Santana Gonzalez et al. 2021(8)

PHASE D: WOLFFIAN DUCTS REGRESSION AND EARLY DIFFERENTIATION

Sex-specific fate of the Wolffian ducts and Müllerian ducts

Initial formation of Müllerian and Wolffian ducts is similar in both male and female embryo before sex determination. Following gonadal differentiation (ovaries in XX female and testes in XY male), the epithelium of the Wolffian ducts involutes in female in the absence of testis-derived androgen (through the action of the transcription factor NR2F2) and the Müllerian ducts regress in male due to the presence of AMH produced by the testes. Differences between male and female embryos is first seen at 10 weeks of gestation in human embryo. In mice, the first histological signs of regression are visible from E14.5 (11).

Besides AMH and its receptors (especially AMHR2 and ALK3), WNT7A, SMAD1,-4,-5,-8, OSX, and CTNNB1 are also required for Müllerian ducts regression (11,15).

The mesonephric ducts remnants in females are represented by the vestigial paroophoron and epoophoron and Gartner's duct (16).

Differentiation: *HOX* genes, retinoic acid, and the Wnt pathway

Specification of the uterovaginal canal into different organ identities is established along the cranio-caudal axis and the organ-specific epithelial morphologies are induced by their surrounding mesenchyme (1). The observation in mice, humans and chicken suggest that the segment-specific identity is likely defined by a spatially coordinated pattern of expression of the posterior *Hox* genes within the mesenchymal cells. In that way, *Hoxa/d9* expression extend more anteriorly in the future oviduct, *Hoxa/d10-11* are limited to the upper and middle segments of the Müllerian ducts, and *Hoxa/d13* are limited to the caudal region of the Müllerian ducts (the anlagen of the cervix and upper vagina). In mice, their expression is detected from E14.5 onwards (8,17). In human fetal samples, *HOXA11* was shown to be expressed in mesenchymal cells of the cranial uterovaginal canal as soon as the 9th week, and in the uterus corpus (but not uterine tubes and cervix) at 11 weeks (7).

Besides *Hox* genes, differentiation of the uterovaginal tract depends, at least in mice, on a cranio-caudal gradient of retinoic acid signaling. Activation (from E14.5 to P0) or absence of the retinoic-acid signaling will result in oviduct/uterine and vaginal fate respectively (18).

Duct patterning also involves the Wnt pathway, especially WNT7A, one of its function being to maintain *Hoxa10* and *Hoxa11* expression (19). Loss of *Wnt7a* in mice results in partial posteriorization of the oviduct and uterus.

Role of ovarian steroids

In primates, there is no evidence for dependance of the early Müllerian ducts organogenesis (i.e., the initiation and elongation steps) to estrogen signaling. In mice, the formation of the uterus is normal in mutants knockout for *Esr1* (estrogen receptor alpha), and/or *Esr2* (estrogen receptor beta). Estrogen responsiveness is, however, required for the maturation and differentiation, the adult uterus displaying hypoplasia and immaturity with fewer glands in *Esr1* mice knockout. In humans, the presence of the estrogen receptors are presumed to be required for the uterine growth, which is most intense between weeks 16 and 24 (20). ESR1 is the dominant estrogen receptor during embryonic development and the principal mediator of estrogen function in the uterus. The onset of *ESR1* expression in the epithelium of the uterovaginal tract varies along the cranio-caudal axis, first detected at 8 weeks of human gestation in the epithelium of the cranial portion of the Müllerian ducts (the future uterine tubes), but later on in the epithelial cells of the solid vaginal plate (from 16 weeks) and not before 21 weeks in the epithelium of the uterine corpus. Expression in the mesenchymal cells was detected from 10-11 weeks onwards in the uterine tubes, from week 12 onwards in precursors of the uterine corpus stroma and from 16 weeks onwards in the vaginal mesenchyme (7,20). *ESR2* is also expressed in the Müllerian epithelia of the uterovaginal tract, detected as early as the 8 weeks of gestation (20). Although absence of *Esr1* and *Esr2* do not induce abnormal morphology of the uterus, exposition to the synthetic estrogen diethylstillbestrol (DES) (discussed in Section III.3) during a window of sensitivity (7-15 weeks of gestation in humans) results in structural uterine anomalies (T-shaped uterus) (20). Alteration of estrogen signaling was shown to alter the *Hox* gene expression pattern, decreasing *Hoxa10* and *Hoxa11* expression, and resulting in a posterior shift in the pattern of *Hoxa*9-11 expression (21,22).

In regards to other steroid hormone receptors, the late expression profile of the progesterone receptor (from 16 weeks in the epithelial cells) and the absence of morphological changes in mice knockout for *Pgr* are against an effect on the organogenesis of the Müllerian ducts. The androgen receptors (AR) are strongly expressed in the mesenchyme at the junction of the vaginal plate and urethra starting at 9 weeks. AR signaling was proposed to influence the position of the Müllerian duct-urogenital sinus junction in mice. In males, the Müllerian ducts-UGS junction is situated in a cranial position secondary to elevated androgen levels. In females, in the absence of androgen action, the junction is more caudal near the vaginal introitus. If this mechanism is also responsible for the difference in location in humans is uncertain (7,23).

Differences in the development of the female genital tract between species

Although the initial formation of the Müllerian ducts is similar and conserved across species, anatomical variation results mainly from differences in the extent of midline fusion of the two Müllerian ducts anteriorly (9,10). Unlike humans, only the caudal part of the Müllerian ducts fuse in mice to form the cervix and the Müllerian vagina. Bilateral oviduct, uterine horns and cervical canals differentiate from the unfused Müllerian ducts. Consequently, the process of fusion and septation taking place in humans is more difficult to characterize and the molecules and pathways involved are mostly unknown so far. Modification in the temporal or spatial expression of *HOX* and/or *WNT* genes likely account for some of the differences in morphology of the female genital tract between species, and explain some irregular uterine anatomy in mice and humans. For instance, in compound *Hoxa13/Hoxd13* mutant mice, the caudal parts of the Müllerian ducts fail to fuse to form the vagina. In humans, pathogenic variants in *HOXA13* result in bicornuate uterus (10).

Differences between humans and mice were also noted in regards to the origin of the adult vaginal epithelium. In mice, it was shown to derive solely from the epithelium of the Müllerian ducts while in humans, the vaginal epithelium is thought to derive from the urogenital epithelium (from the urogenital sinus) (1,24). Finally, differences in the developmental stages in which differentiation occurs (i.e., differentiation for stromal and uterine glandular tissues occurs by 21 weeks of gestation in humans, where it occurs postnatally in mice) can also make comparison between mice and humans difficult.

Table 1.Major genes involved in both uterine and renal development, with existing mouse models.

Gene name	Protein product	Known function in Müllerian Duct development	Known function in nephric ducts/tubules development	Female genital tract phenotype in mutant mice	Renal phenotype in mutant mice	Reference
Formation						
Ctnnb1	β-catenin, Wnt signal transduction factor; cell adhesion	Epithelial cell differentiation, expressed in Müllerian mesenchymal cells	Role in ureteric cell branching	Ctnnb1 activation: hypotrophic uterine horns, coiled oviducts, uterine metaplasia, vaginal imperforation Ctnnb1 deletion: absence of uterus and upper vagina	Overexpression or deletion in the ureteric bud lineage is associated with renal hypodysplasia and renal agenesis	(25–27)
Emx2	Homeodomain Transcription factor	Required in epithelial cells of MD, function unspecified	Required in epithelial cells of WD and mesonephric tubules, function unspecified	Müllerian agenesis	Kidney agenesis, agenesis of Wolffian ducts.	(28)
Gata3	Transcription factor	Müllerian duct elongation Expressed in Wolffian epithelia cells	Ureteric bud positioning	Uterine agenesis with presence of the oviduct	Kidney adysplasia, duplex system, hydroureter	(29)
Greb1l	Possible retinoic acid receptor co-activator	Unknown - Expressed in the intermediate mesoderm	Highly expressed in the fetal kidney	Absence of Müllerian ducts	Renal agenesis	(30)
Hnf1b	Homeodomain transcription factor	Expressed in MD epithelia and in coelomic mesoepithelial progenitors. Involved in cell proliferation, migration, differentiation, epithelial cell polarity	Required in epithelium of WD and for kidney tubule development	Uterine hypoplasia and abnormal differentiation of the epithelium and stroma	Renal agenesis and renal cysts	(31)
Lamc1	Extracellular matrix glycoprotein	Component of the basement membranes. MD elongation?	WD elongation	Uterine aplasia	Renal agenesis	(32)
Lhx1 (Lim1)	Homeodomain Transcription factor	Mesoepithelial progenitor specification Müllerian invagination and elongation	Formation of nephric progenitors, ureteric buds development, renal vesicles patterning	Müllerian agenesis	Renal agenesis	(33–35)

Gene name	Protein product	Known function in Müllerian Duct development	Known function in nephric ducts/tubules development	Female genital tract phenotype in mutant mice	Renal phenotype in mutant mice	Reference
Formation						
Pax2	Paired homeodomain Transcription factor	Mesoepithelial lineage progenitor specification;Müllerian invagination	Nephric lineage specification; required for correct location of UB outgrowth; nephric duct maintenance	Müllerian agenesis	Renal agenesis	(36,37)
Pax8	Paired homeodomain Transcription factor	Mesoepithelial lineage progenitor specification;Müllerian invagination	Nephric lineage specification	Uterine aplasia/hypoplasia and lack of vaginal opening	Hypodysplasic metanephros in $Pax2^{+/-}$ $Pax8^{+/-}$ compound heterozygous	(36,38,39)
Pbx1	Homeodomain transcription factor	MD formation unspecified	Metanephric formation (WD present)	Absence of Müllerian ducts	Renal agenesis/hypoplasia	(40,41)
Retinoic acid Receptors (*Rara,Rarb, Rarg,Rxra, Rxrg*)	Nuclear receptors	Müllerian duct elongation	Pronephros morphogenic field specification, branching morphogenesis	Mouse compound mutants show varying degrees of Müllerian agenesis or hypoplasia	Renal agenesis	(42,43)
Tbx6	T-box Transcription factor	Mesoderm specification	Nephric mesenchyme lineage specification	Vaginal atresia, vaginal duplex (in some background, incomplete penetrance)	Renal and urinary malformation ((in some background, incomplete penetrance))	(44–47)
Wnt4	Secreted Wnt signaling protein	Müllerian invagination Müllerian elongation Myometrium layering, luminal and endometrial gland formation	Mesonephric tubule differentiation; metanephros induction and differentiation	Müllerian agenesis, reduced uterine glands	Rudimentary kidneys	(12,48–50)
Wnt5a	Secreted Wnt signaling protein	Elongation of the lower part Endometrial gland formation	Intermediate mesoderm development Ureteric bud tree development	Absence of cervix and vagina inability to form uterine glands	Duplex kidney and ureters, renal agenesis, hypoplasia, lobularization defects	(50–53)

Gene name	Protein product	Known function in Müllerian Duct development	Known function in nephric ducts/tubules development	Female genital tract phenotype in mutant mice	Renal phenotype in mutant mice	Reference
Formation						
Wnt9b	Secreted Wnt signaling protein	Müllerian duct elongation Expressed in Wolffian epithelia cells	Mesonephric tubules development; metanephros induction and differentiation	Müllerian agenesis	Rudimentary kidneys	(54)
Regression						
Amh	Secreted TGF-B family member	Müllerian duct regression	NR	Persistent Müllerian ducts in males	NR	(55)
Amhr2	Secreted TGF-B family member type 2; Ser-Thr transmembrane receptor	Müllerian duct regression	NR	Ectopic FGT in males	NR	(56)
Wnt7a	Secreted Wnt signaling protein	Müllerian duct regression, differentiation	NR	Ectopic FGT in males	NR	(57)
Differentiation						
Esr1	Steroid nuclear receptor	Müllerian duct differentiation	NR	Hypoplastic uterus Abnormalities of MD development after exposure to exogeneous estrogens (DES), mediated by ERa	NR	(58,59)
Hoxa9	Homeodomain Transcription factor	Patterning upper/middle MD into oviduct	Patterning upper/middle WD into epididymis: nephron patterning; branching morphogenesis	Homeotic transformation	Renal malformation/cysts (in multi-Hox9,10,11 mutant mice)	(60,61)

Gene name	Protein product	Known function in Müllerian Duct development	Known function in nephric ducts/tubules development	Female genital tract phenotype in mutant mice	Renal phenotype in mutant mice	Reference
Differentiation						
Hoxa10	Homeodomain Transcription factor	Patterning middle MD into uterus	Patterning middle WD into vas deferens and seminal vesicle; nephron patterning; branching morphogenesis	Homeotic transformation of uterus to fallopian tube	Renal malformation/cysts (in multi-Hox9,10,11 mutant mice)	(60,61)
Hoxa11	Homeodomain Transcription factor	Patterning middle MD into uterus	Patterning middle WD into vas deferens; nephron patterning; outgrowth of the ureteric bud; branching morphogenesis	Partial homeotic transformation of uterus to fallopian tube	Renal agenesis (homozygous Hoxa11/Hoxd11) Renal malformation/cysts (in multi-Hox9,10,11 mutant mice)	(60–62)
Hoxa13 Hoxd13	Homeodomain Transcription factor	Patterning caudal MD into cervix and upper vagina	Patterning caudal WD into seminal vesicle	Homeotic transformation of cervix to uterus; agenesis of caudal MD	Urinary tract defect/cyst (incomplete penetrance)	(63)
Retinoic acid Receptors (*Rara,Rarb, Rarg,Rxra, Rxrg*)	Nuclear receptors	MD formation and differentiation	Pronephros morphogenic field specification, branching morphogenesis	Mouse compound mutants show varying degrees of Müllerian agenesis or hypoplasia	Renal agenesis	(42,43)
Wnt5a	Secreted Wnt signaling protein	Elongation of the lower part Endometrial gland formation	Intermediate mesoderm development Ureteric bud tree development	Absence of cervix and vagina – inability to form uterine glands	Duplex kidney and ureters, renal agenesis, hypoplasia, lobularization defects	(50–53)
Wnt7a	Secreted Wnt signaling protein	Upper Müllerian tract differentiation into oviducts and uterine horns	NR	Transformation of fallopian tube to uterus and uterus to vagina in females	NR	(19)

Adapted from Santana-Gonzalez 2021, Major 2022, Roly 2018; and the Mouse Genome database. Additional references in the table.
FGT= female genital tract; MD= Müllerian ducts; NR= no defect reported; UB=ureteric bud; WD= Wolfian ducts

3. DEVELOPMENT OF THE RENAL AND URINARY SYSTEM IN HUMANS

Similar to the female genital tract, the urinary system (kidneys and collecting ducts) derives from the intermediate mesoderm. In mammals, three progressively more advanced kidneys arise bilaterally and successively in a cranio to caudal sequence from bilateral stripes of intermediate mesoderm in the trunk of the embryo. The pronephros, composed of 5-7 paired condensations of mesenchymal cells, appears around day 22 (4^{th} week of development) bilaterally in the cervical region of the embryo and connect to the anterior region of the nephric duct (pronephric duct). The pronephros is nonfunctional and do not differentiate further. The pronephric ducts persist, become the mesonephric ducts (the Wolffian ducts) and elongate caudally to reach the cloaca at the 5^{th} week. The Wolffian ducts induce the formation of about 40 pairs of primitive nephrons (the mesonephric tubules) in the adjacent intermediate mesoderm immediately caudal to the last pronephric tubules. Approximately 20 nephrons, located between L1 and L3, continue to differentiate and are transient functional excretory units between the 5^{th} and 12^{th} weeks of development (64,65). During the 5^{th} week, the metanephric mesenchyme forms as bilateral aggregates of cells, derivatives of a more caudal portion of intermediate mesoderm. In their caudal part, the Wolffian ducts give rise to the ureteric buds (at the 28^{th} day of development) as a result of inductive signals from the metanephric mesoderm (66). Orchestrated signaling between the metanephros and the ureteric buds is essential for normal development of the definitive kidneys and excretory systems (67). Branching from the ureteric buds will form the collecting ducts, renal pelvis, ureters and trigone of the bladder. At the end of each branching tips, nephrons are induced in the metanephric mesenchyme. A subset of mesenchymal cells aggregates (the cap mesenchyme (CM)), mesenchymal-epithelial transition of part of the CM forms the renal vesicle, and progression of the renal vesicle into distinct morphological stages then generate the different epithelial segments of the nephron (except for the collecting ducts). The first glomeruli appear at nine weeks (68). Nephrogenesis pursues until the 35-36 weeks of gestation in human (reaching a number of 300.000 up to 1.800.000 nephrons per kidney) (66). Kidneys, first located in the sacral region, ascent to their lumbar position between weeks 6 to 9 (64). The bladder and urethra are a derivative of the urogenital sinus, formed by division of the cloaca at week 4 (64). The urothelium lining the ureter is of mesodermal origin while the urothelium lining the bladder and urethra derive from the endodermal urogenital sinus (7).

Embryological perspective on frequently associated malformations

As discussed later in Section II, renal agenesis is frequently diagnosed in individuals with uterine malformations. Both congenital anomalies may result from defects in shared developmental processes. First, the Müllerian ducts and nephric ducts/tubules share a similar origin (both are derivatives of the intermediate mesoderm (BOX1)). Alteration in formation or patterning of the intermediate mesoderm (i.e., defect in proper programming of progenitor cells) may cause unilateral/bilateral absence of the kidney, uterus and gonads. Second, both organs are formed following cellular processes featuring tubulogenesis (formation of epithelial tubes, epithelial-mesenchymal transition events, processes of invagination, cell migration,…) (10). This may explain that several regulatory genes are essential for both renal and uterine development (highlighted in Table 1). Third, as mentioned above, lack of molecular inductive signals normally secreted by the Wolffian duct (e.g., WNT9B and GATA3) may result in elongation defects of the Müllerian ducts. Recent experiments in mice also demonstrated the contribution of Wolffian mesenchymal cells to mesenchymal tissues in the female oviduct and uterus (69). Finally, frequent co-occurrence of anomalies in the axial skeleton and heart (other derivatives of the mesoderm) or congenital deafness also point to possible defects disrupting major signaling pathways/gradient morphogens (Retinoic acid, WNT, BMP, FGF) involved in the organogenesis of multiple organs (4,70,71).

BOX1: Early stages of embryonic development –from gastrulation to the formation of the intermediate mesoderm

During gastrulation, the one-dimensional blastula transforms into a three-dimensional structure composed of three germ layers: ectoderm, mesoderm, and endoderm. In humans, this process begins around day 15 (equivalent to E6.5 in mice) with the formation of the primitive streak at the posterior side of the embryo. Pluripotent posterior epiblast cells undergo differentiation and ingress through the primitive streak to generate various mesodermal and endodermal derivatives. During the later stages of gastrulation, the posterior epiblast becomes predominantly committed to forming neural and mesodermal tissues, acting as a reservoir of bipotential neuromesodermal progenitors (NMP). Among the mesodermal derivatives is the intermediate mesoderm, a bilateral stripe positioned between the somites and the lateral plate mesoderm in the trunk of the embryo. As the body axis extends, new tissues continuously form at the posterior end. Anterior mesodermal organs form earlier while posterior organs emerge later.

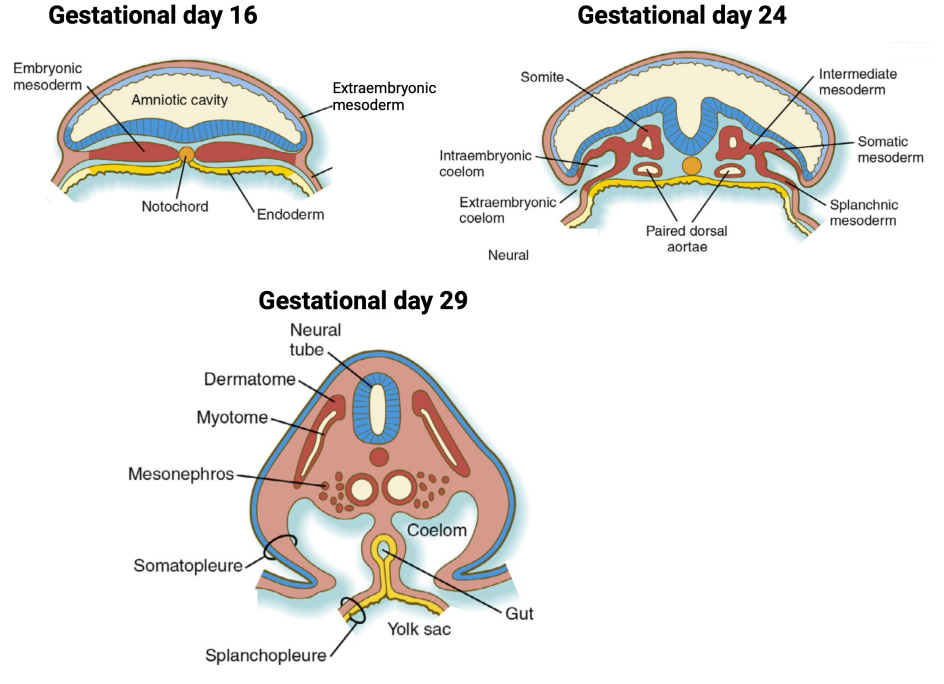


Figure 4. Development of intraembryonic mesoderms
Adapted from Carlson 2015 (72)

The intermediate mesoderm gives rise to key structure of the urogenital system like the Wolffian ducts, Müllerian ducts, nephric mesenchyme (including the mesonephric and metanephric tissues), and the gonads (excluding the primordial germ cells). Lineage tracing studies revealed that the Wolffian duct and nephric mesenchyme have distinct

origins (44). The Wolffian duct primordium arises from the anterior intermediate mesoderm (AIM – Figure 5) before the 7 somites stage (E8.25) in mice, at the level of the 6th somite. Subsequent mesoderm, derived from bipotential neuromesodermal progenitors at the posterior end of the embryo, serves as precursors for the mesonephros, and later the metanephros (from E8.5 to E10.5) (73). The lineage of precursors for the Müllerian ducts, tracing back to the primitive streak, remains undetermined. Given that the duct develops at the level of the anterior mesonephros, it is likely that its progenitors originate from the early posterior intermediate mesoderm (earlyPIM – Figure 5) (73). As the Müllerian duct elongates, additional contributions from more posterior regions of the mesoderm are also expected.

Experiments in animals suggest that the intermediate mesoderm fate and patterning arise secondary to several morphogen gradients (BMP,WNT/FGF; Activin/Nodal; RA)(70).

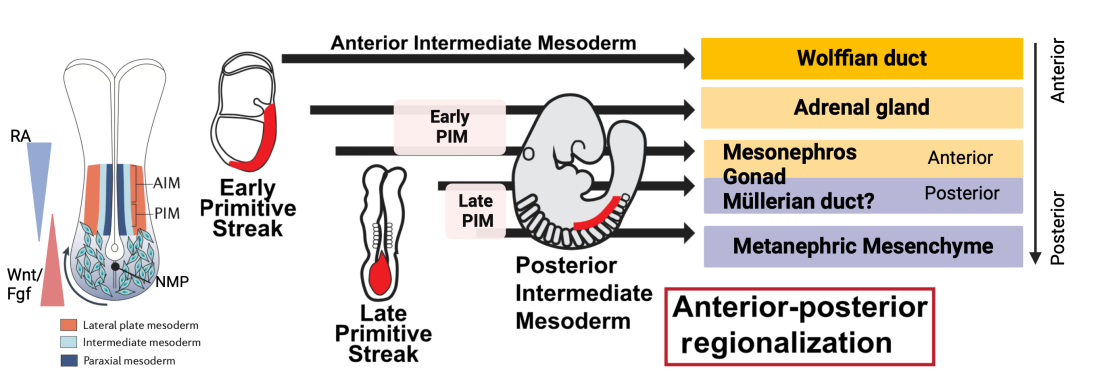


Figure 5. Anteroposterior formation of the intermediate mesoderm
Adapted from Sasaki 2021(73) and from Short 2016 (74)

II. CONGENITAL UTERINE ANOMALIES

Defects in formation, fusion, or septal absorption of Müllerian ducts, or defects in the early process of anatomical differentiation, result in a broad spectrum of uterine malformations. Several classification systems (Supplemental data Table 1) have been proposed for the description of uterine anomalies or other female genital tract (FGT) malformations, for the purpose of guiding diagnosis and therapy (75–78). The European Society of Human Reproduction and Embryology/European Society for Gynaecological Endoscopy (ESHRE/ESGE) classification system groups uterine malformations according to the anatomical components and their corresponding embryological origins (Figure 6) (78). Cervical and vaginal malformations are also classified in this system, but as independent co-existent subclasses, reflecting the fact that these anomalies can be either isolated or manifest in various combinations with different uterine anomalies. Various cervical and vaginal defects, similar to uterine anomalies, may also arise in discrete developmental stages (79). Pelvic MRI is the gold standard to assess Müllerian anomalies, interrogate the presence of Müllerian remnants, the localization of the gonads and the presence of associated kidney and urinary tract anomalies (80,81).

In this second section, we describe the different types of uterine malformations, their clinical impact and their prevalence, as well as malformations in other organs and systems that are frequently associated to uterine malformations.

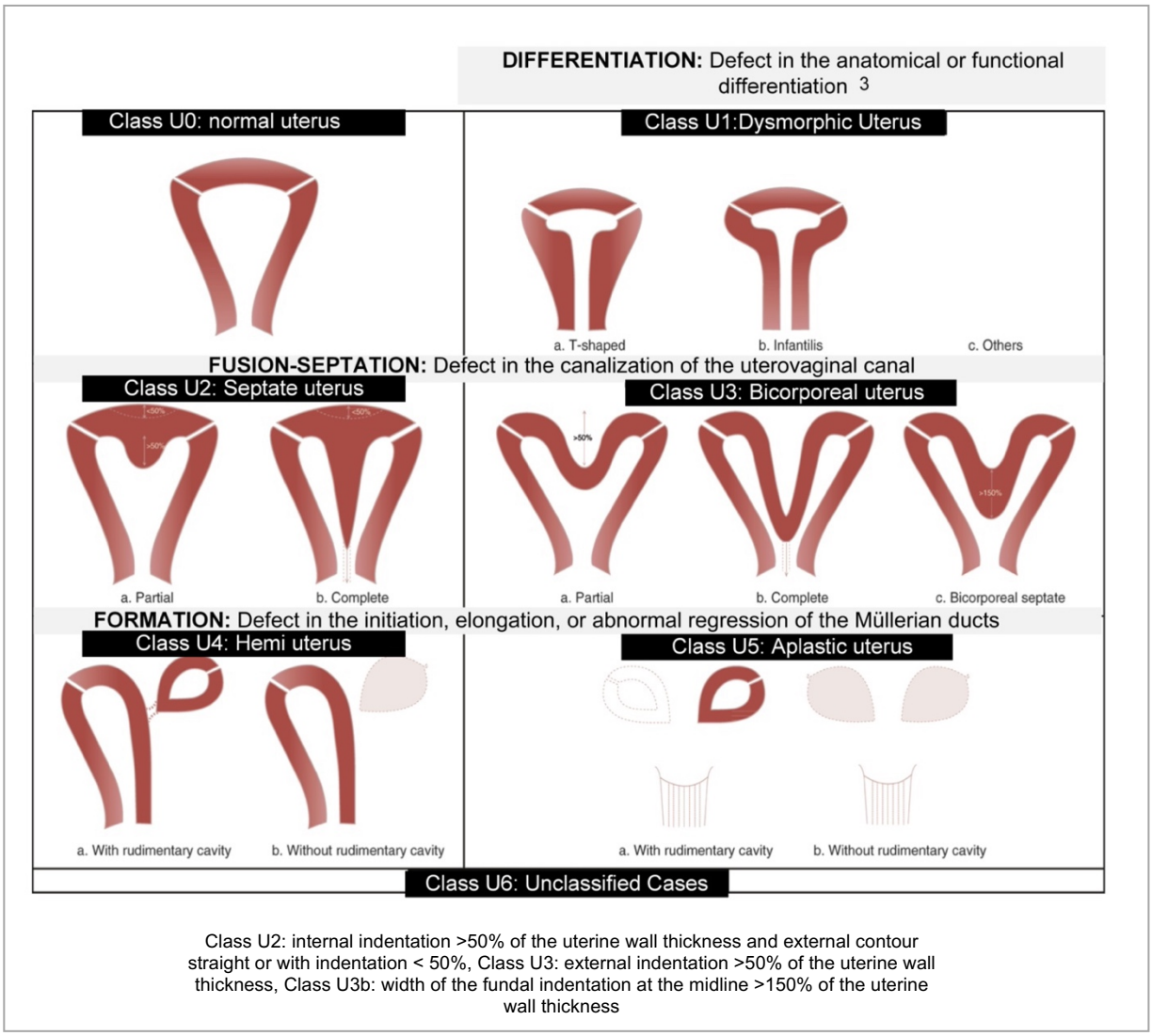


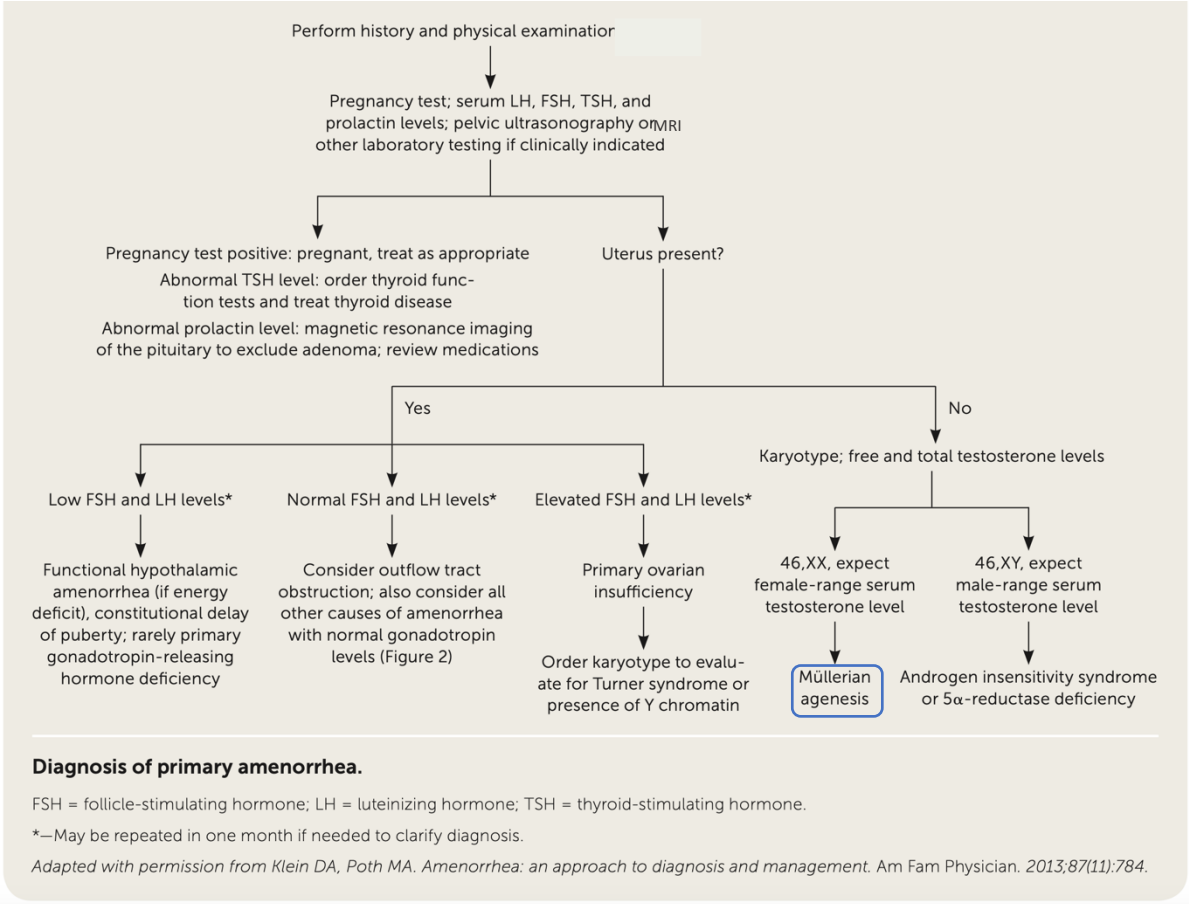
Figure 6. ESHRE/ESGE classification of uterine anomalies.
Classification according to the anatomical components (Class U0 to U6) and their corresponding embryological origins (Formation, Fusion/Septation and Differentiation defects[3]). Adapted from Grimbizis et al., 2013 (78).

1. CLASSIFICATION, SPECIFIC ASSOCIATION AND CLINICAL IMPACT

Uterine malformations occur as a spectrum of variable expression and severity, the most severe being the absence of any fully or unilaterally developed uterus (variably termed aplastic uterus, uterus aplasia, uterine agenesis, or congenital absence of the uterus; class U5 in ESHRE/ESGE classification), with or without rudimentary cavities (unilateral or bilateral horns/uterine remnants). An aplastic uterus is usually diagnosed during late adolescence because of primary amenorrhea, with or without pain. The

[3] The term "anatomical differentiation" underlies the process by which the identity of the Müllerian duct epithelia/mesenchyme is determined to be organ specific (embryonic and fetal life).
The term "functional differentiation" is the process by which the phenotype of the epithelia changes during estrous or menstrual cycles (82)

diagnostic approach in front of primary amenorrhea (Figure 7) will include pelvic ultrasonography or MRI and in 15% of the cases, Mayer-Rokitansky-Küster-Hauser syndrome will be diagnosed (83). Mayer–Rokitansky–Küster–Hauser syndrome (MRKH; MIM# 277000 and 601076; U5C4V4 in the ESHRE/ESGE classification) described the association of the congenital absence of the uterus, cervix, and the upper part of the vagina in an otherwise phenotypically normal 46,XX female. Fallopian tubes are frequently normal but may be hypoplastic/aplastic or malformed (84). Persistence of at least the fimbriated end of the fallopian tube is typical and unexplained to date. The structure and function of the ovaries are usually normal although gonadal dysgenesis and ovarian agenesis have been described in a few cases (85). Ectopic ovaries have been reported in up to 40% of individuals (86). Adrenarche and thelarche are typically normal and FSH and LH in the normal range for the age (with however higher LH/FSH ratio levels) (87). In the literature, MRKH syndrome is also referred as Müllerian agenesis, Müllerian aplasia, or congenital absence of the uterus and vagina and is generally divided in two categories. Type 1 MRKH (complete or typical form) features a complete absence of uterus and vagina without any associated malformations. Completely asymptomatic individuals with uni- or bilateral vestigial remnants of Müllerian tissue that do not contain any endometrial tissue are still categorized as Type 1. Type 2 MRKH (incomplete or atypical form) has either partial absence of uterus and vagina and/or additional associated malformations (frequently renal and spinal malformations; less frequently cardiac, ocular, auricular anomalies, and inguinal herniae (85)). Individuals with small remnants of Müllerian tissue, which are symptomatic because of the presence of some functioning endometrial tissue, are categorized as Type 2. MURCS (Müllerian, renal, and cervicothoracic somite) association represents one specific subtype of type 2 MRKH (88). In regards to treatment, primary nonsurgical dilatation, and more rarely surgical procedures in addition, are options for vaginal elongation (80). Surrogate pregnancy or adoption were the only options available to MRKH patients desiring children until recently. Since 2014, human uterine allotransplantation (UTx) has emerged as a possibility of childbearing. It was estimated that around 80 UTx have been performed in the world in women with absolute uterine factor infertility (the vast majority being affected by MRKH syndrome) between 2014 and 2021, resulting in the birth of more than 40 children (89). Finally, psychological support is essential. Diagnosis at the time of adolescence, a period shaped by the development of female and sexual development, may represent for the patient a great emotional and mental burden, due to the impact on sexual life and future childbearing abilities (83).



Diagnosis of primary amenorrhea.

FSH = follicle-stimulating hormone; LH = luteinizing hormone; TSH = thyroid-stimulating hormone.

*—May be repeated in one month if needed to clarify diagnosis.

Adapted with permission from Klein DA, Poth MA. Amenorrhea: an approach to diagnosis and management. Am Fam Physician. *2013;87(11):784.*

Figure 7. Diagnostic approach in primary amenorrhea.
From Klein et al. 2019 (90).

Other uterine malformations include hemi-uterus (unicornuate uterus, uterus unicornis; class U4 in ESHRE/ESGE classification), partial or complete bicorporeal uterus (also referred in the literature as bicornuate or bifid uterus) and bicorporeal septate uterus (class U3 in ESHRE/ESGE classification), and partial or complete septate uterus (class U2 in ESHRE/ESGE classification).Other terms that have been used in the literature are the terms Herlyn–Werner–Wunderlich syndrome and OHVIRA (obstructed hemivagina, and ipsilateral renal agenesis) syndrome which describes both the specific association of a complete bicorporeal uterus with a double cervix (didelphys uterus) and a longitudinal obstructing vaginal septum associated with renal agenesis ipsilateral to the obstructed hemivagina (91). All these anomalies may lead to medical or obstetric complications such as miscarriage, prematurity, fetal malposition, dystocia, low birth weight in offspring, or acute abdominal pain (e.g., from hydrometrocolpos), when they are associated with any cervical or vaginal obstructive anomaly (92–95). Many uterine malformations may be totally asymptomatic and undiagnosed unless they are specifically sought.

Finally, the ESHRE/ESGE classification includes two other classes of uterine anomalies. Dysmorphic uteri (class U1 in ESHRE/ESGE classification) such as infantilis uterus or T-shaped uterus have been reported after prenatal drug exposure (discussed in section III) and when untreated, are frequently associated with unfavorable fertility and pregnancy outcome (96). Infantilis uteri may be in some cases secondary to ovarian dysgenesis (e.g., as in Turner syndrome) and have also been reported in other syndromic conditions. The category of unclassified uterine malformations (class U6 in ESHRE/ESGE classification) covers combined pathologies or extremely rare malformations such as a duplication defect, which can be a feature of the caudal duplication syndrome (OMIM# 607864).

BOX2: Phenotypic variability across uterine anomalies study cohorts

Cohorts of women with uterine anomalies that have been studied for prevalence, recurrence, or genetic etiologies of congenital uterine anomalies have usually included admixed types of uterine anomalies (mainly U2, U3, U4, and U5, as described above), or have focused on Mayer–Rokitansky–Küster–Hauser syndrome (U5C4V4 according to the ESHRE/ESGE classification). The extra-uterine phenotypes are also usually variable within most study cohorts, including some individuals with isolated uterine anomalies, others with a complex, non-isolated phenotype (typically with involvement of kidneys or vertebrae), and sometimes those with uterine malformations in the context of a recognizable Mendelian genetic syndrome. Splitting the different classes of malformations is necessary when determining the prevalence for each group, as it is highly variable according to the severity of the anomalies observed, or in terms of clinical management. When interrogating etiologies or recurrence, considering all classes of uterine malformations may be of interest. In fact, the observation of defects involving different classes in some families (97–105) suggests that similar genetic background could still predispose to different types of uterine malformation with variable penetrance and expressivity .

2. PREVALENCE

The prevalence of uterine malformations has been estimated to be as high as 5.5- 9.8% in a general female population and increasing up to 25% in women with a history of infertility or miscarriage (106,107). These estimates include the relatively high frequency of arcuate-shaped uteri (3.9- 6.8%). Though this configuration is considered by some as a normal variant rather than a true malformation, it does reflect a minor degree

of a septation defect (88). The meta-analysis of Chan et al. [2011](106), involving a total of 5,163 women, reported a prevalence of 2.3% for partial and complete septate anomalies (canalization defects), a prevalence of 0.7% for bicorporeal uteri (0.4% for bicornuate uteri and 0.3% for didelphys uteri), and 0.1% for hemi-uterus (unicornuate uteri). MRKH syndrome is rare, with a prevalence of 1 in 4,500–5,000 live female births (108,109).

3. ASSOCIATED MALFORMATIONS

Congenital anomalies of the kidney and urinary tract (CAKUT) are one of the most frequently associated group of malformations. Although the incidence in the general population is 1/500 live births (0.2%), CAKUT was identified in 20% (+/- 9%) of individuals with MRKH (2,85,110–112) and in up to 40% of individuals with uterine malformations in European studies (2). Renal agenesis is the most frequent type of CAKUT, reported in 4.7-13% of individuals with uterine agenesis, and with higher frequency (70%) in individuals with didelphys uterus (2).

Malformations of the axial skeleton, including idiopathic scoliosis, and in some studies *spina bifida*, are the second most common anomalies, diagnosed in 21-32% of individuals with uterine malformations. Scoliosis (idiopathic or congenital) is the most frequent anomaly, reported in 11-20.3%. Fusion defect (Klippel-Feil anomaly, fusion of caudal vertebrae) is identified in 0.5 to 3.2% (85,110,112).

As already mentioned, ovaries are found in ectopic location in up to 40% of individuals with Müllerian anomalies (2).

Following endocrinological workup, hyperandrogenemia was diagnosed in 48.3% of individuals with MRKH, with no increased incidence of polycystic ovary syndrome compared to the general population. However, only a low percentage (3.2%) presented clinical signs of acne or hirsutism. The causes are not well known. Only a few numbers of cases can be explained by genetic variation in *WNT4* (113). Missing crosstalk regulation between ovaries and the uterus was suggested as a possible physiopathological mechanism (87,113).

Other malformations that are less frequently associated affect the heart (1.8-2%) and/or ears (0.6-4%). Anal atresia, absent radius, gonadal dysgenesis, tubo-ovarian agenesis, congenital ocular malformation, inguinal/umbilical hernia are each reported in no more than 1% of cases (85,112).

Finally, dysmorphological assessment in a cohort of 115 individuals with MRKH syndrome confirmed the absence of specific dysmorphic features and significant abnormalities in growth parameters (105).

III. ETIOLOGIES OF CONGENITAL UTERINE ANOMALIES

1. MODE OF INHERITANCE AND RECURRENCE

The etiology of most uterine malformations is generally believed to be complex (i.e., multifactorial). Recurrence risk for first-degree relatives is estimated at 1–5%, and the few existing studies (Table 2) have established a similar risk for siblings to most multifactorial disorders. Numerous reports of discordant phenotypes in monozygotic twins (e.g., MRKH syndrome vs. normal uterus) do not support a strong heritability and suggest that post-zygotic somatic mutations or non-genetic mechanisms such as epigenetic or microenvironmental factors may underlie the occurrence of congenital uterine malformations (101,114–120). However, whereas these mechanisms may explain sporadic cases, reports of familial cases do support the existence of a predisposing genetic background that may be stronger in some families compared to others. A recent report documented the first case of MRKH syndrome being transmitted from mother to daughter through surrogate pregnancy, linked to a 4Mb *de novo* deletion on chromosome 2q37 (121). According to Hammoud et al. [2008], 10% of causality might be attributed to familial affiliation, a term they use to encompass shared genetic and environmental factors in families. They reported a 12-fold increased risk for first-degree relatives of patients affected by Müllerian duct anomalies (MDA), and a detectable increased risk in second and third-degree relatives, although the latter was not statistically significant (104). Moreover, it is likely that the role of inherited factors and the true rate of familial recurrence have been underestimated in most of existing studies. Uterine ultrasounds were usually not performed, or not performed in extended family members and therefore asymptomatic or subtle anomalies were unlikely to be ascertained. On the one hand, it may be clinically appropriate to ascertain recurrence only for those anomalies that are functionally/clinically obvious, as this is what patients may be most concerned about; but on the other hand, it is necessary to consider subtle anomalies as part of the spectrum occurring within families in order to truly understand the genetic contribution. In most families, recurrences tend to recapitulate the same type of uterine malformation, but recurrences with variable types of uterine maldevelopment also have been reported in several families (97–105,109). Consequently, it may not be possible to predict the severity of a recurrence based on the phenotype of a proband. Finally, reports of a large number of families in the literature with recurrence of Müllerian and kidney anomalies or Müllerian and axial skeletal anomalies suggest that radiological investigation of these systems should be considered in relatives before calculating genetic contribution in research

(99,100,102,122–126). Estimates of recurrence for clinical purposes ideally should also include risk estimates for renal anomalies. The term hereditary urogenital adysplasia was suggested to describe families with associated renal and uterine malformations, which features autosomal dominant inheritance with variable expressivity (partially sex-dependent), and incomplete penetrance (122).

BOX3: The concept of incomplete penetrance and variable expressivity

Individuals with the same genetic disorder may exhibit different malformations within the same system (e.g; different types of uterine malformation or CAKUT), variation in regards to which systems are affected, or differences in symptom severity. This variability among individuals from the same family or with the same genetic condition is known as variable expressivity. Penetrance, on the other hand, refers to the proportion of individuals with a particular genotype who actually show the expected clinical symptoms or develop the disease. For a given condition, penetrance is considered reduced or incomplete if some individuals with the genotype do not display any symptom by a specific age (127). Incomplete penetrance can explain why unaffected parents may transmit pathogenic variants to affected offspring. Causes of variable expressivity and incomplete penetrance may involve the polygenic background (the cumulative effect of small-effect genetic variants), contribution of environmental exposures, as well as epigenetic factors and stochastic events at the tissue level that may modify the expression of the causal gene. When considering uterine malformation, the penetrance may also be sex-dependent and only manifests in female individuals.

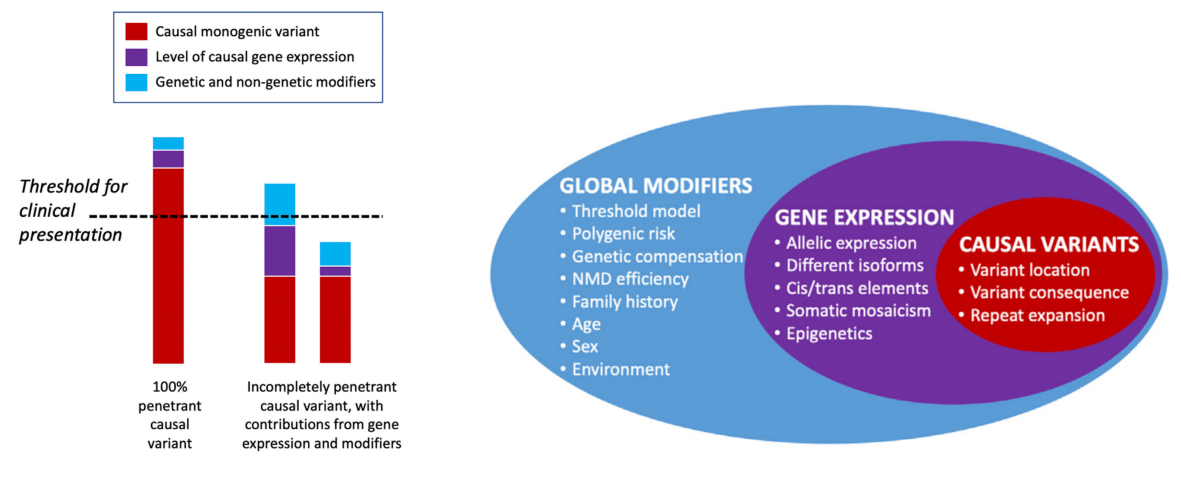


Figure 8. The treshold model of disease . Monogenic variant may only display a phenotype when additional genetic or non-genetic factors are present. From Kingdom 2022 (127).

Table 2. Studies evaluating recurrence of urogenital or uterine malformations in families of patients with uterine malformations.

Study	*Number of probands*	*Anomalies*	*Relatives studied*	*% individuals with a family history of urogenital malformation**	*Recurrence risk of urogenital malformation for siblings/ relatives of a woman with MRKH/Congenital Uterine Anomalies*	*Method*
Brucker 2020	129	MRKH	First degree relatives	13/129 **(13%) (U/R/G)**	NA	Questionnaire and digitized medical records
Rall 2015	246[a]	Typical MRKH (n=184) Atypical MRKH (n=143) MURCS syndrome (n=19)	Fiirst degree relatives	39/246 **(15%) (U/R)**[b]	NA	Questionnaire
Hammoud 2008	1376[c]	High probability of Mullerian anomalies (ascertainment limited to billing and ICD codes)	First and second degree relatives	19/1376 **(1.4%) (U)**	**Relative risk (U):** First degree relatives: 11.6 (p<0.001) Parent/children: 8.78 (p<0.001) Siblings: 12.98 (p<0.001)	Kinship analysis from a large population database
	1397		Family clustering (≥5 affected descent)	256/1397 **(18%)(U)**	First cousins: 1.44 (p=0.29) Second cousins: 1.30 (p=0.11)	
Wottgen 2008	59[a]	Typical MRKH (n=45) Atypical MRKH (n=12) MURCS (n=16)	Siblings	1/59[d] **(1.7%)(U/R)**	**Siblings: 0.97% (1/103 siblings) (U/R)**	Questionnaire
van Lingen 1998[g]	34	MRKH	Siblings	1/34 **(2.9%) (U)**	**Female siblings : 2.9% (1/34 post-pubertal sisters) (U)**	Observations in context of molecular studies
Petrozza 1997	58[e]	MRKH (surrogate pregnancies)	Offspring	NA	**Offspring: 0% (0 (U/R)**/17 females and 0 (R)/17 males)	Questionnaire
Pavanello 1988	20 (11)[f]	MRKH	First-degree relatives	4/11 **(36.4%) (U/R)**	**Siblings: 3.2% (+-1.8%)** [e] **(U/R)**	US examination
Elias 1984	24	Bicorporeal uterus (n=9) Hemi-uterus (n=2) Septate uterus (n=11) Arcuate uterus (n=2)	First and second degree relatives	1/24 **(4.2%) (U)**	**Female siblings: 2.7%** (1/37) (female siblings/**U**)	Interviews
Carson 1983	23	Aplastic uterus (n=22) Other uterine malformation (n=1)	First and second degree relatives	0/23 **(0%) (U)**	**Female siblings: 0%** (0/30 post-pubertal sisters) **(U)**	Interviews

MRKH: Mayer-Rokitansky-Küster-Hauser syndrome (U5C4V4 in the ESHRE/ESGE classification); MURCS: müllerian, renal, and cervicothoracic somite syndrome; NA: data non available.

*Evaluation of recurrence for uterine (U), genital (G) or renal abnormalities (R).

[a] Number of individuals out of the total number who had siblings *and* completed the questions with respect to siblings.

[b] This number also includes individuals with a family history of skeletal malformations or other malformations.

[c] 1397 – 21 = 1376 (subtraction of 21 individuals in order to consider only one proband per family when there are multiple affected family members).

[d] Occurrence of unilateral testis in 1 sibling was not included because a primary gonadal malformation indicated distinct developmental pathways, and was probably unrelated to MRKH in the affected proband.

[e] Represents the total number of probands in the study. However, the number of women who actually had offspring is not available.

[f] 20 probands were included but only 11 sibships were investigated by ultrasound. The number of siblings for these 11 families was not available. The recurrence risk for siblings to be affected with any type of Müllerian or renal defect is probably underestimated because it refers to the total 20 sibships.

[g] Referred to in Simpson, 1999.

2. GENES INVOLVED IN CONGENITAL UTERINE ANOMALIES

The primary approach to identify genes potentially involved in human uterine malformations has started with hypotheses regarding candidate genes based on animal models, followed by targeted assays (usually Sanger sequencing and more recently Next generation sequencing (NGS)) in affected and control populations, in an effort to prove or disprove an association. Additional proposed candidates have been based on known gene-gene interaction networks. Monogenic multiple malformation syndromes that can involve the uterus have also been a source for potential candidates for isolated uterine malformations. Finally, recurrences of several copy number variants found in 10-14% of affected individuals (125,128–130) have suggested interesting candidate genes. With the advent of next generation sequencing, targeted analysis of multiple candidate genes at once in case and control cohorts brought additional evidence for the involvement of several candidate genes. Finally, whole exome sequencing (WES) led to the discovery of novel candidate genes based on a hypothesis-free approach.

In total, more than 50 genes have been suggested as candidates for MRKH syndrome (31,131). However, only three genes (*WNT4, HNF1B* (and 17q12 deletion) and *GREB1L*) are related to MRKH in the Online Mendelian Inheritance of Man database (OMIM) so far, gathering sufficient evidence to consider definitive/strong causality. Interestingly none of these genes have been strongly associated with isolated MRKH (MRKH type 1), as individuals had in most of the cases additional renal anomalies (*HNF1B* and *GREB1L*) or hyperandrogenism (*WNT4*). For *HNF1B* and *GREB1L*, the mode of inheritance is autosomal dominant with incomplete penetrance and variable expressivity. Heterozygous *WNT4* variants are assumed to be associated with autosomal dominant inheritance, although the few reported cases were all sporadic.

Only the genes that have been tested in cohorts of patients with MRKH and/or uterine malformations are reviewed in details in this section, starting with the genes that have gathered a high level of evidence and ending with genes with limited or contradictory evidence. Categories are based on the evidence-based framework developed by the Clinical Genome Resource (ClinGen Curation guidelines) (Figure 9) (132), with the limitation that this classification was optimized for monogenic conditions with Mendelian inheritance, and may not be perfectly suitable for conditions with a more complex mode of inheritance (such as oligogenic or multifactorial diseases). Genes in the same pathway will be discussed together even when they do not all have the same level of evidence (the gene with the highest level of evidence was considered for the classification of the pathway).

Candidate genes that have been interrogated by targeted sequencing in cohorts of individuals with uterine malformations, and the number of variants identified, are also detailed in Supplemental data Table 2.

Supporting evidence to validate the causality of a gene for a specific human disease include gene-level evidences and variant-level evidences. Experimental data that support the role of the gene in the disease (gene-level evidence) include expression and functional data (e.g., interaction with protein known to be involved in the disease, expression in tissues relevant for the disease, biochemical function consistent with the phenotype) as well as *in vivo* modelling (e.g., disruption of the gene in animals or organoids leading to a phenotype consistent with the human disease, or rescue of the phenotype following addition of the wildtype gene). The variant-level evidence is based on the identification of several disrupting variants in the gene in several individuals with the specific condition. Variants that will be considered convincing must be rare in the general population (variable according to the mode of inheritance) and predicted deleterious for the function of the protein (truncating variants leading to haploinsufficiency or loss-of-function; missense variants predicted *in silico* to be damaging). For several of the genes considered below, gene burden analysis (confirming the enrichment of variants in the gene in cases compared to controls), and functional studies (confirming the deleterious effect for the missense variants identified in single cases) are often missing to prove definitely an association.

The genes detailed below are associated with non-syndromic, or mildly syndromic, congenital uterine anomalies. Most of them can be assigned to two group in regards to their function: (I) genes encoding transcription factor or coactivator and (II) genes encoding signaling molecules (WNT signaling, BMP signaling)) (Table 3). Reports of uterine malformations in several syndromes (Supplemental data Table 3) suggest that other pathways/groups of protein (e.g., extracellular matrix proteins, protein involved in ciliogenesis) are likely important for uterine morphogenesis, although their exact roles are currently unknown.

Table 3. Genes that have been associated with non-syndromic (or mildly syndromic) congenital uterine anomalies

Transcription factors
Homeodomain transcription factors : *EMX2, HNF1B,LHX1, PAX2, PAX8, PBX1, HOXA10, HOXA11, HOXA13*
T-box transcription factor : *TBX6*
P53 family of transcription factor: *TP63*
Nuclear hormone receptor: *ESR1, RARA*
Coactivator: *GREB1L*
Signaling molecules
Wnt signaling pathway: *DACT1, WNT4, WNT5A, WNT7A, WNT9B*
BMP-SMAD signaling pathway: *BMP4, BMP7, AMH*
Structural protein : *LAMC1*
Endonuclease : *GEN1*

Evidence Level		Evidence Description
Supportive Evidence	DEFINITIVE	The role of this gene in this particular disease has been repeatedly demonstrated in both the research and clinical diagnostic settings, and has been upheld over time (in general, at least 3 years). No convincing evidence has emerged that contradicts the role of the gene in the specified disease.
	STRONG	The role of this gene in disease has been independently demonstrated typically in at least two separate studies providing **strong** supporting evidence for this gene's role in disease, usually including both of the following types of evidence: • Strong variant-level evidence demonstrating numerous unrelated probands with variants that provide convincing evidence for disease causality[1] **as well as** • Compelling gene-level evidence from different types of supporting experimental data[2]. In addition, no convincing evidence has emerged that contradicts the role of the gene in the noted disease.
	MODERATE	There is **moderate** evidence to support a causal role for this gene in this disease, typically including both of the following types of evidence: • Several probands with variants that provide convincing evidence for disease causality[1] • Moderate experimental data[2] supporting the gene-disease association The role of this gene in disease may not have been independently reported, but no convincing evidence has emerged that contradicts the role of the gene in the noted disease.
	LIMITED	There is **limited** evidence to support a causal role for this gene in this disease, such as: • Fewer than three observations of variants that provide convincing evidence for disease causality[1] OR • Variants have been observed in probands, but none have sufficient evidence for disease causality. • Limited experimental data[2] supporting the gene-disease association The role of this gene in disease may not have been independently reported, but no convincing evidence has emerged that contradicts the role of the gene in the noted disease.
NO REPORTED EVIDENCE		Evidence for a causal role in disease has not been reported. These genes might be "candidate" genes based on linkage intervals, animal models, implication in pathways known to be involved in human diseases, etc., but no reports have directly implicated the gene in human disease cases.
Contradictory Evidence	CONFLICTING EVIDENCE REPORTED	Although there has been an assertion of a gene-disease association, conflicting evidence for the role of this gene in disease has arisen since the time of the initial report indicating a disease association. Depending on the quantity and quality of evidence disputing the association, the association may be further defined by the following two sub-categories: 1. **Disputed** a. Convincing evidence *disputing* a role for this gene in this disease has arisen since the initial report identifying an association between the gene and disease. b. Refuting evidence need not outweigh existing evidence supporting the gene:disease association. 2. **Refuted** a. Evidence refuting the role of the gene in the specified disease has been reported and significantly outweighs any evidence supporting the role. b. This designation is to be applied at the discretion of clinical domain experts after thorough review of available evidence
NOTES		
[1]Variants that disrupt function and/or have other strong genetic and population data (e.g. *de novo* occurrence, absence in controls, strong linkage to a small genomic interval, etc.) are considered convincing of disease causality in this framework. [2]Examples of appropriate types of supporting experimental data based on those outlined in MacArthur et al. 2014.		

Figure 9 .ClinGen Clinical Validity Classifications and Qualitative Descriptions. From Strande et al., 2017 (132).

A. Genes and pathways with definitive level of evidence for causality

HNF1B

HNF1B (hepatocyte nuclear factor-1B; also known as *TCF2*) is a POU-homeodomain-containing transcription factor. Gene inactivation in mouse embryos is lethal before the gastrulation stage (133). *Hnf1b* has been shown to be strongly expressed in Wolffian and Müllerian ducts during development (134), and is involved in processes of cell proliferation, migration and differentiation as well as in epithelial polarization of the Müllerian ducts (31) . The gene positively regulates *Lim1*, *Pax2*, and *Wnt9b* expression in mice (133), three genes associated with partial or complete absence of the FGT when knocked out in mice (135). Conditional knockout of *Hnf1b* in the epithelial cells of the Müllerian ducts resulted in hypoplasia and abnormal differentiation of the uterus, with, in a subset of the mutant mice, associated kidney anomalies (renal agenesis, cysts), mirroring MRKH type 2 (31). In Humans, *HNF1B* deficiency is associated with renal cysts and diabetes syndrome (RCAD syndrome; MODY type 5, OMIM#: 137920) a multisystemic condition in which the pancreas and kidneys are the most frequently affected organs. However, the renal cysts and diabetes are not always present and other organs (e.g., liver, pancreas) may also be affected. Uterine malformations are a frequent finding in RCAD syndrome with congenital uterine anomalies identified in up to 40% of females with *HNF1B* point mutations or deletions (136). The mode of inheritance is autosomal dominant with highly variable expressivity. Although a family history of renal anomalies or diabetes will be highly suggestive, absence of family history does not rule out *HNF1B* deficiency as 50% pathogenic variants arise *de novo* (137).

Since the first report of MRKH in two sister with kidney disease and diabetes (138) in 1999 , about 20 unrelated individuals with syndromic and variable uterine malformations have been reported in the literature with heterozygous *HNF1B* point mutations. Notably, all of these individuals, except one, have presented additionally with renal disease and/or diabetes. Besides point mutations, deletions in 17q12 encompassing *HNF1B* and *LHX1* were identified in 2-9% of women with typical or atypical MRKH anomaly (31,128,130,139–142) and in 1.85% in a cohort of individuals with Müllerian fusion defects (143) . In Oram et al. [2010], the prevalence of *HNF1B* mutations was 16%

(8/50 patients)[4] in women with both uterine (the most prevalent being the bicorporeal uterus class) and kidney abnormalities (144). However, *HNF1B* sequencing in series of patients with isolated uterine anomalies or in unselected cohorts of individuals with MRKH type 1 and 2 did not uncover any mutations (124,139,140,144,145).

Overall, *HNF1B* variations seem to be rarely associated with isolated uterine anomalies in the absence of renal abnormalities (in either the affected individuals or their relatives), but testing should be considered in individuals with a personal or family history of renal anomalies or diabetes.

GREB1L

First associated with CAKUT in 2017, *GREB1L* heterozygous variants were also identified in individuals with congenital uterine anomalies (CUA) and MRKH syndrome in some of the reported families (30,146,147). Published in 2020 (see the Results Section), our work supported causality of the gene in families with hereditary urogenital adysplasia and in individuals with MRKH syndrome type 2 (148). The mode of inheritance is autosomal dominant with incomplete penetrance, and a bias towards maternal inheritance has been proposed (149). Recently, an unbiased rare variant enrichment analysis in two independent cohorts of individuals with MRKH syndrome identified predicted heterozygous deleterious variation in *GREB1L* in 16 individuals/590 (2.7% of the two cohorts) (149). Eight probands presented MRKH syndrome type1, bringing evidence to broaden the phenotypic spectrum of *GREB1L* mutations to isolated uterine malformations. Besides renal and uterine malformations, the *GREB1L*-associated disease includes less frequently inner ear anomalies (e.g., cochlear agenesis and sensorineural hearing loss) and skeletal malformations in isolated or variable associated clinical presentation without clear genotype-phenotype correlation highlighted so far (Figure 10) (148–150). Finally, complex congenital heart diseases (e.g., tetralogy of Fallot, hypoplastic left heart syndrome, left ventricular hypertrophy, aortic stenosis) have been reported in a few individuals (150).

[4] One individual was removed from the total considered in the publication as the variant p.(Val61Gly) is now classified benign.

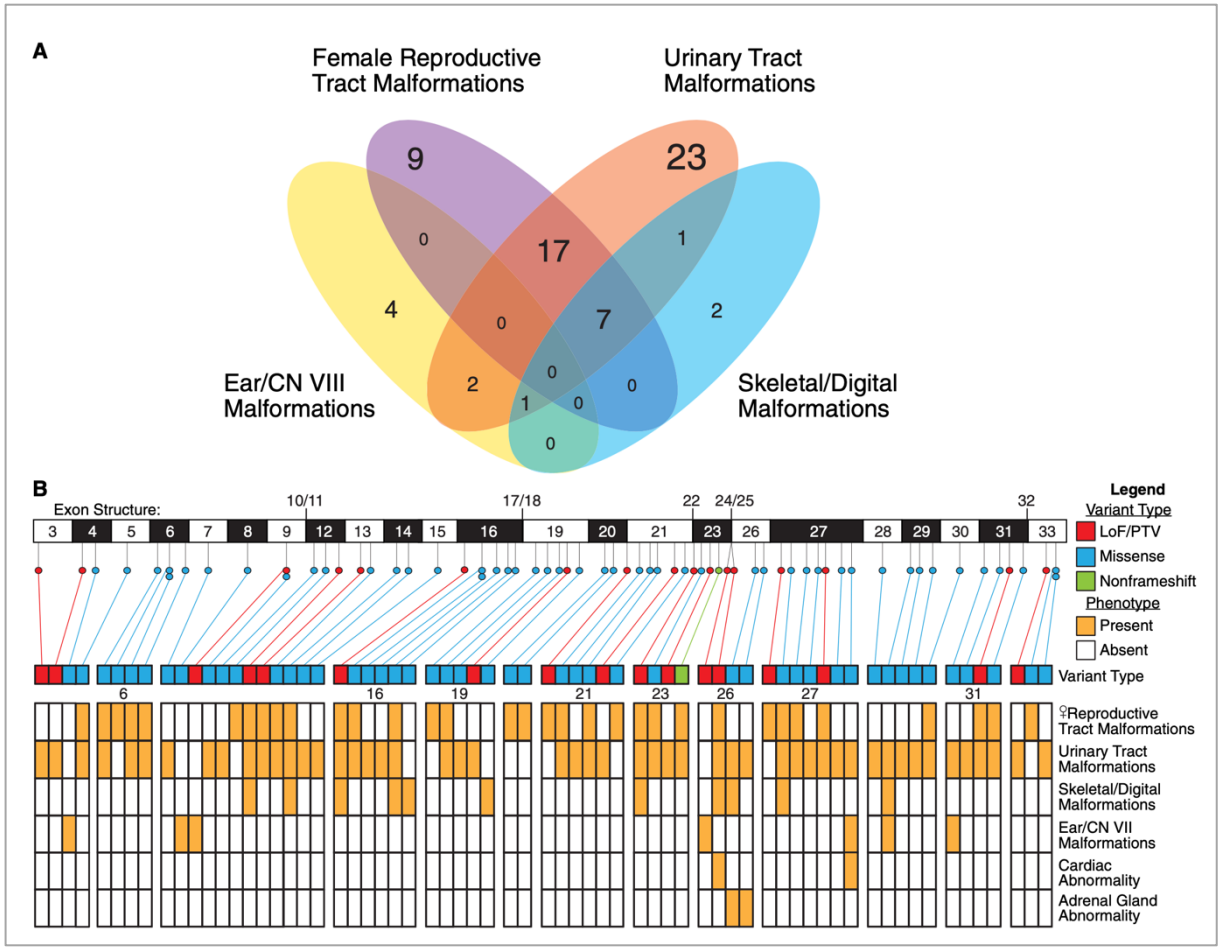


Figure 10. *GREB1L*-associated malformations.
The phenotypic spectrum (and frequency of malformations) associated with *GREB1L* heterozygous variants overlap with the spectrum of malformations in MRKH syndrome type 2. No clear genotype-phenotype correlation does explain the variable expressivity (From Jolly 2023) (149).

The function of GREB1L and its role during kidney and female genital tract development is not yet understood. *GREB1L* (for growth regulation by estrogen in breast cancer 1 like) is a retinoic-acid responsive gene (151,152) and encodes a protein shown to be part of a chromatin complex with retinoic acid and steroid hormone receptors (153). It has been suggested to act as a coactivator in retinoic acid-mediated transcription (154) and may play roles in cell differentiation, ribosome biogenesis, and protein O-GlcNAcylation (155). In mice embryo at E8.5, *Greb1l* is expressed in the neural tube, endoderm, posterior paraxial mesoderm and lateral plate mesoderm (155). It is a marker of mesodermal differentiation within neuromesodermal progenitors in the tailbud (156). At later stage (E16), the gene is expressed in the liver, thymus, intestine, kidney and brain (30). In human adult tissues, the expression levels were found to be highest in the vagina, cervix, and epididymis (30). During kidney development, *Greb1l* is expressed in nephron during early and late nephrogenesis (30,157). Animal models have confirmed its causality in

renal and uterine agenesis. Knockout mouse embryos at E13.5 lack kidneys and genital tracts, with remnants of mesonephric tubules but no metanephros, Wolffian, or Müllerian ducts (30). In zebrafish, pronephric defects have been observed (147). Additional abnormalities in mutant mouse embryos include exencephaly, reduced body size, abnormal heart morphology (crisscross heart malformation), and lethality between E14.5 and birth (P0) (30,155).

HOX Genes

During embryonic development, transcription factors encoded by the conserved homeobox (HOX) genes act to determine the anteroposterior patterning of several organs (Figure 11). HOX genes are clustered on four chromosomes in mammals. The most 5' genes of clusters A and D, *Hoxa9–13* and *Hoxd9–13*, are expressed in developing Müllerian ducts in mice (135). Following an anteroposterior axis of expression, *Hoxa-9* is most expressed in the fallopian tubes, *Hoxa-10* in the uterus, *Hoxa-11* in the uterus and uterine cervix, and *Hoxa-13* in the upper vagina (17). *Hoxd9–11* are expressed in the uterus during development with a similar pattern of expression (61) and *Hoxd13* in the caudal portion of the Müllerian ducts (63). Functional redundancy is a key feature of the HOX genes family and transformation of one single gene in a cluster may be compensated by a paralog. In the murine uterus, redundancy in function as well as synergistic effects have been observed for flanking Hox genes (*Hoxa9–11*) and for paralogous genes from different clusters (*Hoxa9–11* and *Hoxd9–11*). Partial and complete anteriorization of the uterus—meaning a homeotic transformation to a morphology and histology resembling oviducts—can be observed in *Hoxa10* and *Hoxa11* knockout mice, respectively (158,159). A more severe phenotype was noted in mice with flanking null mutations simultaneously introduced for *Hoxa9–11*, which was further worsened when accompanied by flanking heterozygous mutations introduced for *Hoxd9–11* (61). Flanking homozygous loss of *Hoxd9–11* on its own, however, is not sufficient to cause a uterine phenotype. *Hoxa13* knockout mice lack the caudal portion of the Müllerian ducts (63). *Hoxd13*$^{-/-}$ female mice were shown to be fertile (internal urogenital organs not described) (160), but compound *Hoxa13*$^{+/-}$ *Hoxd13*$^{-/-}$ mice were sterile with uterine and/or vaginal abnormalities (partial agenesis of uterus and vagina, septated vagina), renal and rectal abnormalities (63). *Hoxa9–13* and *Hoxd9–13* are also particularly important in vertebrate limb development (161) and several Hox genes are expressed in the developing kidney, including *Hoxa9–11* and *Hoxd9–11* (162).

Pathogenic variants in *HOXA13* are associated with hand–foot–genital (HFG) syndrome (OMIM# 140000). HFG syndrome is characterized by fully penetrant limb malformations of variable severity, and partially penetrant urogenital defects, which can include defects of Müllerian fusion and septal absorption to a variable degree (163). Missense variants result in a more severe phenotype compared to a presumed loss-of-function mechanism with frameshift variants, gene deletion, and polyalanine expansions (164), and hence a possible dominant negative or gain-of-function effect has been suspected. Interestingly, a female individual with bicorporeal uterus, left arm agenesis and bicuspid aortic valve was recently reported with heterozygous predicted deleterious missense variants in both *HOXA13* and *HOXA9*, possibly explaining the more severe phenotype (163). Finally, *HOXA13* has not been associated with MRKH phenotypes so far.

Less evidences are currently available to support causality of other *HOX* genes. Rare variants of uncertain significance have been identified in *HOXA10* and *HOXA11* in 12 individuals with sporadic uterine malformations. Further epidemiological or biological evidence is needed before concluding that any of these variants affects uterine development.

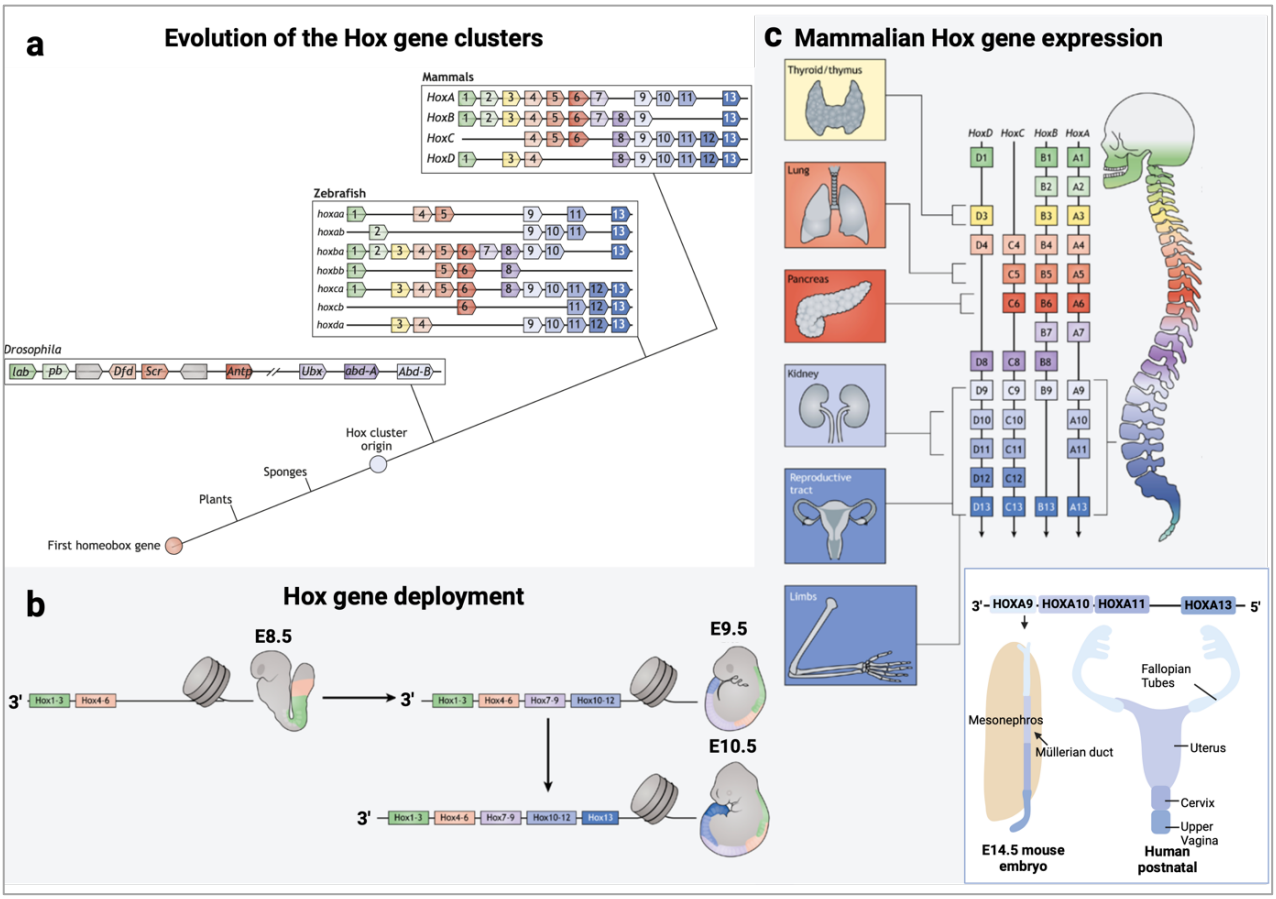


Figure 11. The Hox code.
a. Hox genes are organized into four chromosomal clusters in mammals and seven in zebrafish.
b. Epigenetic regulation of the hox gene clusters control their strict spatiotemporal expression. In vertebrates, the expression of hox genes starts at gastrulation with progressive activation, following their 3' to 5' genomic location (referred as temporal collinearity), in a posterior growth zone as the body axis elongates.
c. The hox signature is transmitted from the progenitors (mesoderm and neuromesodermal progenitors) to their progeny, allowing a spatial distribution of the patterning information in various axial tissues and the limbs (spatial collinearity). In addition, gene- and tissue-specific regulatory events may further refine Hox expression domains in axial tissues (165,166). (Illustration adapted from Hubert 2023, Created in BioRender.com)

B. Genes and pathways with strong level of evidence for causality

WNT4 and the Wnt Signaling Pathway

Wnt pathways control several basic embryonic developmental processes including body axis patterning, cell fate specification, cell proliferation, and cell migration. It has long been recognized as one of the principal signaling pathways during development of mammalian urogenital tracts, mainly through the Wnt/β-catenin signaling pathway with particular contributions from WNT9B and WNT4 (54,167). During the first step of

Müllerian duct development, invagination of coelomic epithelial cells requires WNT4 signaling and its absence of expression leads to Müllerian duct agenesis in both male and female mouse embryos (48). *Wnt4* is expressed by mesenchymal cells as well as by cells in the coelomic epithelium alongside the elongating Müllerian ducts (8,12), and is furthermore essential for proper elongation of the ducts and in the process of their epithelialization during embryogenesis. Later on, WNT4 is critical for proper myometrium layering and for luminal and endometrial glands formation (12). *Wnt9b*, is expressed by Wolffian duct cells, and paracrine signaling from these cells is necessary for Müllerian duct elongation. *Wnt9b* knockout female mice lack the oviduct, uterus, and upper part of the vagina (54). Gonads are normal in *Wnt9b*$^{-/-}$ mice, whereas ovaries in *Wnt4*$^{-/-}$ female mice are abnormal with a testis-like appearance and abnormal expression of steroidogenic enzymes. Both genes are furthermore involved in the induction of metanephric kidneys as knockout mice develop rudimentary kidneys. WNT9B acts upstream of WNT4 and is likewise indispensable for mesonephric tubule development (54). Later nephrogenesis, medullary differentiation, and collecting duct development involve WNT9B, WNT4 as well as additional WNT ligands and receptors from the Wnt pathway (i.e., WNT11, WNT7B, and WNT5A). *Wnt5a* homozygous mutations in mice have been implicated in duplex collecting system malformations via the non-canonical Wnt/PCP signaling pathway (53). In uterine development and differentiation, two other secreted proteins of the Wnt pathway have been shown to play an important role. WNT7A, secreted by Müllerian duct epithelial cells, is involved in the patterning of Müllerian ducts. Posteriorization of the FGT is noted in *Wnt7a*$^{-/-}$ mice, usually with absence of oviducts and a vaginal-like histological appearance of the uterus (168). WNT5A, secreted by mesenchymal cells, is necessary for posterior development of the FGT as knockout mice lack cervical and vaginal structures (51). Both WNT7A and WNT5A are furthermore involved in uterine gland development within the endometrium (135). *WNT* genes participate in the development of other organs as well. For example, *Wnt7a* plays a role in limb development and *Wnt9b* knockout mice present variably penetrant cleft lip/palate.

Based on phenotypic similarities with the knockout mice model, *WNT4* was first sequenced in women with MRKH syndrome presenting biochemical and clinical features of hyperandrogenism. Four individuals (all presenting MRKH syndrome type 2 and hyperandrogenism) with heterozygous loss-of-function missense variants in the gene were identified by Biason-Lauber and Philibert between 2004 and 2011. They demonstrated *in vitro* that the variants failed to inhibit steroidogenesis in ovarian cell lines and decreased the level of ß-catenin, alone or in combination with the wildtype, supporting a dominant

negative effect (169–171). Parental analyses of the probands were only partially performed for two individuals, and did not identify the variant in the mothers. Based on these results and the data in animals, *WNT4* was the first gene from the Wnt pathway to be associated with MRKH syndrome in humans. However, following sequencing in larger cohorts of individuals with MRKH or uterine malformation (supplementary data Table 2), *WNT4* variants were only rarely identified (two additional synonymous variants and five missense variants of unknown significance identified in total) suggesting that the prevalence of *WNT4* variants is likely low in unselected cases (131,172–174). Around 25 additional missense variants are reported in Clinvar[5]. Most of them are variants of unknown significance underlining the need for more systematic functional analyses in order to facilitate the interpretation and genetic counseling. Further characterization of the endocrine phenotype of a larger number of individuals with *WNT4* variants would help to determine if hyperandrogenism is a mandatory clinical criterion to suspect *WNT4* defect. Finally, the absence of variants in many individuals with MRKH and hyperandrogenism suggest that other proteins in the WNT4/ß-catenin signaling pathway could be involved although no strong candidate has been identified so far (113,175). Finally, heterozygous *WNT4* variants have also been reported in individuals with renal agenesis or hypodysplasia (176,177), and one homozygous variant was found in three fetus siblings with female sex reversal, dysgenesis of kidneys, adrenals, and lungs (a condition named SERKAL syndrome) (178).

WNT9B has gathered limited evidence for causality. Targeted sequencing/analysis of the gene in cohorts with MRKH syndrome (131,173,179,180), or CUA (181–183) has identified one loss-of-function variant, two nonsense variants (Non-mediated decay not predicted to occur) and nine variants of unknown significance (Supplemental data Table 2). The phenotypes were bicorporeal uterus for one individual, MRKH type 1 for 8 individuals and MRKH type 2 for two individuals. Waschk et al. suggested that *WNT9B* might lead to uterine malformation possibly in a polygenic manner as they identified co-inheritance of other genetic factors (*TBX6* variant or *LHX1* deletion) in two individuals (182). Digenic inheritance was also suggested by Wang et al. who identified co-inheritance of heterozygous *GEN1* variants in two individuals. In one of the two families, segregation analysis showed inheritance of the frameshift *WNT9B* variant from the unaffected mother whereas the *GEN1* variant was inherited from the father. In the other family, the unaffected mother and sister were only carrier of the *GEN1* variant (father not tested).

[5] Clinvar is a freely accessible archive of genomic variation identified in humans and their relationships to human health, with supporting evidence (https://www.ncbi.nlm.nih.gov/clinvar/).

The double heterozygous *Gen1*$^{+/-}$*Wnt9b*$^{+/-}$ mice presented abnormal development of the uterus (longer length) compared to controls (183). Finally, homozygous *WNT9B* variants were recently reported in two families with bilateral renal agenesis/hypodysplasia. Non-penetrance is described in one unaffected sibling carrier of the homozygous nonsense variant (184).

Amongst other genes of the Wnt pathway, *WNT7A, WNT5A* and *DACT1* have been sequenced in cohorts of individuals with non-syndromic CUA without any convincing results so far.

WNT7A homozygous mutations were first associated in 2006 with a range of limb malformations, including the phenotypes of Al-Awadi/Raas-Rothschild (AARR) syndrome (characterized by apparent phocomelia, hypoplastic pelvis, and abnormal genitalia) and Fuhrmann syndrome (considered an attenuated phenotype of AARR) (185). Two patients with AARR syndrome and different *WNT7A* homozygous missense mutations in exon 4 were reported with uterine hypoplasia (186,187). The uterine phenotype was unknown or not reported for other affected female individuals (185,186,188) and several individuals with uterine anomalies were reported with AARR syndrome or Schinzel phocomelia (a syndrome controversially grouped with AARR syndrome in OMIM) before the gene discovery (189–193). Beyond this easily recognizable condition, *WNT7A* has been tested in three series of patients with MRKH or Müllerian anomalies (173,194,195) with no evidence so far for causality. Only two rare heterozygous synonymous variants (not predicted to alter the splicing by SpliceAI[6]) were found in two individuals with isolated Müllerian anomalies.

WNT5A heterozygous variants are associated with Robinow syndrome in humans (OMIM 180700), a condition characterized by short stature with limb shortening, dysmorphic facial features and sometimes cardiac and genitourinary malformation. Uterus malformation has not been reported in syndromic cases. Among 189 females with non-syndromic uterine malformations, one individual with a bicorporeal uterus was identified with an intronic variant of uncertain significance in *WNT5A* which was absent in 198 controls (196).

Finally, DACT1 is a cytoplasmic protein interacting with DVL proteins which are central mediators of the canonical and non-canonical Wnt pathway. *Dact1* knockout mice display posterior defects including lack of vagina and uterine hydrometra, renal agenesis or dysplasia, anorectal malformation and caudal vertebrae agenesis (197). In humans,

[6] SpliceAI uses deep neural networks to predict whether splicing events occur. The score can range from 0 to 1, where a higher score indicates a stronger probability that the variant will disrupt normal splicing.

DACT1 heterozygous variants were recently associated with CAKUT and additional features reminiscent of Townes-Brocks syndrome. One of the reported individuals presented bicorporeal uterus (OMIM# 617466) (198,199). The gene was sequenced in one cohort of individuals with uterine malformations, identifying rare missense variants of unknown significance in two individuals (200). However, segregation analysis and functional analysis were not performed and *in silico* data rather predict benign effects for these variants.

TBX6

TBX6 encodes a transcription factor characterized by a T-Box DNA-binding domain. The gene is involved in the specification of the mesoderm in early development and plays a role in patterning of the somites, left/right patterning, as well as in the determination of the nephric mesenchyme cell fate in mice (44,45,201). Besides vertebral and rib abnormalities, some homozygote hypomorphic mutations in *Tbx6* are also associated with urogenital malformations with incomplete penetrance (45,46). Depending on the genetic background, up to 60% of mice were affected with kidney malformations (renal aplasia/hypoplasia, malrotated, or displaced kidneys, hydronephrosis) or genital malformations (vaginal atresia or vagina duplex in females).

Based on these observations in animal models, *TBX6* appeared the most likely susceptibility gene for uterine malformations in the region 16p11.2. Deletion in 16p11.2 (600 kb -BP4-BP5) has been identified in 1-6% of individuals with MRKH (128,130,142,180) which is a much higher frequency than the 0.03-0.04% prevalence observed in the general population (202,203). Enriched prevalence of this copy number variation (CNV) is also found in cohorts ascertained for intellectual disability/multiple congenital anomalies (0.33%), autism spectrum disorder (0.7-1%), morbid obesity (0.7%), CAKUT (0.3%) and congenital scoliosis (7.5%) (204–209). By targeted sequencing (130), and more recently WES (180,210), heterozygous single nucleotide variants were identified in individuals with MRKH syndrome and further supported the involvement of *TBX6* in uterine malformations. In a Chinese MRKH cohort, the prevalence of *TBX6* variants was 3.3% (14/422) compared to 0.6% (6/1038) in female controls (p=.0004, odds ratio=5.25). Following sequencing in an additional cohort of individuals (American/European and Chinese background) and *in vitro* analyses, Ma et al. could confirm loss of function effects through different mechanisms for 8 variants identified in 8 individuals/622 (1.3%). The uterine malformation was isolated, or associated with scoliosis/vertebral malformation or renal hypoplasia (210). Incomplete penetrance in familial cases and the low frequency in

heterozygous carriers of 16p11.2 deletion suggest that haploinsufficiency of one *TBX6* allele is likely insufficient to cause MRKH syndrome and that penetrance of the uterine phenotype may result from a more complex mechanism (210).

In humans, pathogenic variants in *TBX6* are also known to cause a spectrum of segmental vertebral defects, from the most severe spondylocostal dysostosis (SCD, OMIM#122600) to congenital scoliosis, the severity of the phenotype probably depending on the severity of the loss of *TBX6* function (211). Although one single familial case was reported with autosomal dominant spondylocostal dysostosis and a possibly dominant negative stop-loss mutation in the gene (212), the mode of inheritance is autosomal recessive in the majority of the cases (211,213). Change in *TBX6* dosage (below 50%) is necessary for penetrance of the phenotype (214). Biallelic missense variants have been reported in patients with SCD while biallelic null alleles, resulting in the absence of *TBX6*, are believed to cause embryonic lethality, similar to findings in mice (211). Combination of a damaging variant and the noncoding mild hypomorphic T-C-A haplotype (i.e., co-occurrence of the three single nucleotide polymorphism (SNP [rs2289292 (T), rs3809624 (C) and rs3809627 (A); common among European (33%) and Asians (44%)) was identified as a frequent cause for milder segmentation vertebral defects and congenital scoliosis, explaining around 10% of the cases (213,215–217). A compound mode of inheritance with 16p11.2 deletion and the same hypomorphic haplotype was identified in six patients with skeletal and kidney dysplasia, and was suggested to explain the incomplete penetrance in one family with CAKUT (218,219). Finally, reduction in dosage of *TBX6* below haploinsufficiency was shown to increase the penetrance of renal malformation in mice (47). If this genetic mechanism is also causal for the penetrance of the uterine phenotype is unknown so far, but no additional risk haplotypes were identified with deleterious *TBX6* variants by Ma et al. in individuals with MRKH syndrome (210). One previous Finnish study reported two single nucleotide variants (rs56098093 (p.(Gly162Ser); and rs201231713 (p.(Arg272Gln))) with a statistically significant higher frequency in females with MRKH (130)(13.4% compared to 4% in controls (p-value 0.0021), and 9.8% compared to 1% in controls (p-value 0.0002) (130).The two variants are located within the conserved T-Box domain and predicted to be damaging (The REVEL scores[7] are Deleterious (Supporting) (0.67), and Deleterious (Moderate) (0.89) respectively). Functional analyses

[7] REVEL score: is an ensemble method for predicting the pathogenicity of missense variants based on a combination of scores from 13 individual tools: : MutPred, FATHMM v2.3, VEST 3.0, PolyPhen-2, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP++, SiPhy, phyloP, and phastCons. The REVEL score for an individual missense variant can range from 0 to 1, with higher scores reflecting greater likelihood that the variant is disease-causing (220).

would be required to determine if these relatively frequent single nucleotide polymorphism could represent hypomorphic variants. In their studies, Tewes et al. (221) did not confirm a higher frequency of rs56098093 compared to their control cohort.

C. Genes and pathways with moderate level of evidence for causality

PAX8

PAX8 is a transcription factor of the paired-box family that has been associated with isolated and syndromic thyroid dysgenesis (222). Deletion in the chromosomal region 2q14.1, including *PAX8*, were identified in two girls presenting with hypothyroidism early in life and uterine malformation (MRKH in one, hemi-uterus in the other) (223,224). *Pax8* homozygote knockout mice, supplemented with synthetic T4, are infertile due to uterine aplasia/hypoplasia and lack of a vaginal opening (38). During murine development, *Pax8* expression is detected in epithelium of the uterus, oviduct, and vagina. PAX8, with PAX2, is also a central regulator of kidney development and acts upstream of LIM1 and GATA3 (39). More recently, by comparing the prevalence of *PAX8* variants in individuals affected by MRKH and controls, Chen et al. [2021] identified *PAX8* as one of the most significant gene, underlying the etiology in 1.2% of individuals (7 loss-of-function heterozygous variants/592) (180). Hypothyroidism was an inconsistent feature. In addition, three missense variants of unknown significance were also identified in this cohort as well as one additional loss-of-function variant in a patient with congenital hypothyroidism, found to have MRKH syndrome after ultrasound examination of the pelvis. Renal agenesis has also been reported in individuals with syndromic *PAX8* mutations (222).

D. Genes and pathways with limited level of evidence for causality

LHX1

As discussed above, the rare pathogenic deletion in the region 17q12 was found in several MRKH/CUA- affected individuals with higher frequency compared to controls (128,142,202). Besides *HNF1B*, the region also includes *LHX1*, another interesting gene for Müllerian anomalies. Involvement of *LHX1* in uterine malformation is supported by studies in animal models. *LHX1* encodes a LIM-homeodomain transcription factor and its

expression is likely essential in the Müllerian duct epithelial progenitor cells for ductal elongation and uterine endometrium differentiation. Loss of expression leads to shortened oviducts, uterine aplasia, and infertility in Müllerian duct-specific Lhx1 conditional knockout mice (33). Downstream target genes are currently unknown which limits functional analysis of candidate variants. From six series of individuals with MRKH or CUA, six missense variants (including one of them recurrent in three individuals) of uncertain significance and one truncating variant have been identified in *LHX1* by targeted sequencing or WES (130,139,140,145,225–227). However, no functional analyses was performed, except for one variant with confirmation of decreased transcriptional activity (227).

EMX2

EMX2 encodes a homeobox-containing transcription factor expressed in urogenital epithelial tissues, the dorsal telencephalon, and the olfactory epithelium during development. Female knockout mice lack the genital tract, kidneys, and gonads (28) and also present with brain malformations. *EMX2* expression has been shown to be repressed by *HOXA10* (228) and one of its target genes might be *TP63* (229). Liu et al.[2015] demonstrated expression of a transcript in human adult uterus (NM_004098.3; NP_004089.1) and sequenced the gene in 517 women with uterine malformations (fusion or septation defects) and 563 controls (229). One heterozygous nonsense variant, resulting in a truncated protein lacking the homeodomain, was identified in an individual with a complete bicorporeal uterus with a double cervix (didelphys uterus). A dominant negative effect for this variant was suspected following functional studies on transfected human embryonic kidney cells (229). Finally, remnants of Müllerian ducts were found in a male with a 10q26 deletion including *EMX2* (230).

PAX2

Loss of the transcription factor gene *Pax2* (paired box gene 2) in homozygous knockout mice causes agenesis of kidneys, ureters, and genital tracts (37). *Pax-2* is expressed in both Wolffian and Müllerian ducts (37). However, it has not been determined yet if absence of female reproductive tracts result from defective action of PAX2 in the Müllerian ducts or if this disturbance is secondary to the abnormal Wolffian duct development. Among 192 individuals with various uterine malformations, only one rare synonymous variant was identified in one individual with a complete bicorporeal uterus

and absence of fallopian tubes (231), and one heterozygous missense variant was found in a patient with MRKH type 1 in a cohort of 111 individuals with MRKH syndrome. Moreover, the SNP rs12266644 was associated with Müllerian duct anomalies with statistical significance compared to controls in Chinese Han females (p=0.008) and increases the risk for MDA in the dominant model (p=0.015, OR:1.637 95% CI:1.096-2.443) (232). In humans, *PAX2* mutations are associated with papillorenal syndrome and isolated renal hypoplasia (233). Increase in *PAX2* mRNA, and hypomethylation within the promotor of the gene were detected in endometrial tissue from septate uterus compared to tissue from controls, suggesting that epigenetic modification and aberrant *PAX2* expression might contribute or be associated with Müllerian anomalies (234).

BMP signaling

Heterozygous loss-of-function variants in *BMP4* and *BMP7* were identified in four individuals with MRKH type 1 and one individual with MRKH type 2 (presenting an idiopathic scoliosis besides the uterine phenotype) (180), which reached significance for mutational burden (False discovery rate adjusted p=0.03 for both *BMP4* and *BMP7*).

BMP4 and *BMP7* have been shown to be important for many steps of kidney development but their role in Müllerian duct development has not been investigated yet. Mouse embryos homozygous for null mutations in *Bmp4* are lethal between E6.5 and E10. *H*eterozygous mice display ectopic ureteric bud formation, ureterovesical junction-type hydronephrosis, double collecting system and hypo/dysplastic kidneys, besides other malformations (e.g., polydactyly, microphtalmia, craniofacial defects) (235). At the time of ureteral budding and subsequent rounds of branching (at E10.5-12.5), *Bmp4* is exclusively expressed in the stromal mesenchymal cells surrounding the Wolffian ducts and the stalk of the ureteric bud, but not in the metanephric mesenchyme surrounding the tip of the ureteric bud. In contrast, *Bmp7* is expressed in both the ureteric epithelium and the metanephric mesenchyme and is required for nephron progenitors' self-renewal. Knockout *Bmp7* mice display kidney hypodysplasia (236). Although *Bmp4* and *Bmp7* promote the growth and elongation of the ureter and the development of the metanephric mesenchyme, low BMP activity is necessary at the ureter budding site. In mice, *Grem1* was shown to antagonizes *Bmp4* and *Bmp7* in a spatiotemporal manner around the ureteric bud tip and branching tips, guiding ureteric bud outgrowth from the Wolffian duct and enabling subsequent branching (237). In regards to Müllerian duct formation, BMP signaling was shown to be required in chicken for early specification of the mesoepithelial progenitors,

formation of the placode in the coelomic epithelium, and induction of *PAX2* and *LHX1* expression (238).

GEN1

GEN1 (encoding a Holliday Junction endonuclease) was shown to regulate ureteric bud formation and branching during metanephric organogenesis in mice and to increase expression of *Gdnf* and retinoic-acid signaling molecules (239,240). Mice homozygous for partial loss-of-function mutations were fertile but displayed kidney and urinary tract defects with incomplete penetrance. Following the detection of one heterozygous deletion including *GEN1* (chromosomal region 2p24.2) in one individual with MRKH type 1, sequencing of the gene was pursued in a cohort of 125 individuals with Müllerian anomalies, identifying 4 missense variants and one nonsense in 5 individuals. Two subjects also presented a heterozygous *WNT9B* variant suggesting possible digenic inheritance. The authors investigated the possibility of synergistic effect of both variant in double heterozygous mutant mice, showing that they presented longer uteri with disorganized stromal structure (183).

E. Genes and pathways with conflicting evidence reported

CFTR

CFTR encodes a chloride channel which controls ion and water secretion and absorption in epithelial tissues. Variants in the gene are responsible for cystic fibrosis and CFTR-related disorders with autosomal recessive inheritance. The possibility that *CFTR* defects may cause MRKH syndrome was considered given the involvement of biallelic variation in congenital bilateral absence of the vas deferens (CBAVD, part of the CFTR-RD phenotypes). The hypothesis was that the defect could be secondary to developmental anomalies of the Wolffian ducts, which may consequently impact the development of the Müllerian ducts. This hypothesis is not supported by observation in mice embryo, showing that CBVAD in CFTR-related disorder is secondary to a progressive involution of the vas deferens rather that an early defect in Wolffian duct formation (241,242). Targeted analysis of *CFTR* in 111 women with MRKH identified 9 heterozygous variants in the gene, but no biallelic variations (131). In addition, no variants were identified in a series of 25 patients (194). Besides the absence of embryological evidence for Müllerian duct

anomalies in mice models (8), the absence of biallelic variants in MRKH cohorts does not support a causative role for *CFTR* in MRKH syndrome. Involvement of heterozygous variation seems moreover unlikely given the high frequency of *CFTR* heterozygous carriers in the Caucasian population (around 1/25) and the rarity of MRKH syndrome (1/4500).

F. Candidate genes

Candidate genes suggested by animal models and targeted sequencing

Retinoic acid receptors

Retinoic acid is required at multiple steps of uterine formation. Observation in vitamin A deficient embryo and RAR mice mutants indicated a role in early formation of the rostral and caudal Müllerian ducts, and later on for midline fusion, and differentiation. Redundancy was also demonstrated with developmental defects only when several RA receptors were mutated (243,244). Müllerian ducts were totally absent at E12.5 when all *Rara* isoforms and the *Rarβ2* isoform were inactivated ($Rara^{-/-}Rarβ2^{-/-}$) or at E14.5 in $Rara^{-/-}Rxra^{-/-}$ double mutants. Only the caudal part (body of the uterus and upper part of the vagina) was absent, with incomplete penetrance, in mice compound knockout for $Rara1^{-/-}/Rarβ2^{-/-}$ or $Rara^{-/-}Rarγ^{-/-}$, as well as in compound mutants for $Rxrα^{-/-}$ and either $Rara^{+/-}$,$Rarβ^{-/-\ or\ +/-}$, $Rarβ2^{-/-}$ or $Rarγ^{-/-\ or\ +/-}$ (243,244). Defects in Müllerian duct formation were unlikely to be the consequence of primary defect in Wolffian ducts formation as Wolffian ducts were formed in $Rara^{-/-}/Rarβ2^{-/-}$ double mutants. RAR double mutants also presented renal agenesis/hypoplasia and ectopia, and RA signaling was shown to be required for ureteric bud formation and growth of the metanephric blastema. Moreover, RA signaling is active in the stroma of Müllerian ducts from E14.5, with a decreasing gradient necessary for proper differentiation. High level of retinoic acid in the cranial region determines the oviduct and uterine fate and absence of retinoic acid signaling in the caudal part is necessary for differentiation into vagina (18).

In a cohort of 111 individuals with MRKH, two rare missense variants were identified in *RARA* in one proband with MRKH type 1 (it is however not specified if the variants were in *cis* or *trans*). One additional heterozygous variant was identified in the same cohort in another individuals with MRKH type 1 (131).

AMH

The Anti-Müllerian Hormone (AMH), also called Müllerian-inhibiting substance (MIS), is produced after gonadal differentiation by the Sertoli cells in the fetal testes, and in males, induces regression of the Müllerian ducts. Pathogenic variants in *AMH* are associated, in males, with an autosomal recessive condition leading to the persistence of Müllerian derivatives (uterus and tubes) in otherwise normally virilized males (OMIM#261550). Based on the hypothesis that activating mutation in *AMH* or its receptor *AMHR2* could result in abnormal Müllerian duct regression in females, sequencing of these genes was performed in series of individuals with MRKH syndrome (131,245,246). Three heterozygous missense variants of unknown significance were identified in *AMH* amongst 111 affected individuals. However, no functional analyses were performed to validate their possible damaging effect (131). In an association study in a Han Chinese population, the authors reported that the variant c.934C>T (p.Arg312Cys) in *AMH* was protective in regards to isolated MRKH (p=0.039,OR=0.346,95%CI=0.115-1.043) (247). Replication will be necessary to prove this association, with consideration given to the potential contribution of ethnic background.

LAMC1

LAMC1 encodes an extracellular matrix glycoprotein, component of the basement membrane. Mice knockout for the gene, present defect in Wolffian ducts elongation, with anomalies of the basement membranes, and abnormal blind-ending Müllerian ducts resulting in Müllerian aplasia and renal agenesis (8,32). Targeted analysis of exome sequencing data in a cohort of 111 individuals with MRKH syndrome identified four rare heterozygous missense variants of unknown significance in the gene (131).

ESR1 and OXTR

ESR1 encodes for the estrogen receptor-1 and *OXTR* for the oxytocin receptor. Methylation and expression studies in DNA extracted from tissue of uterine remnants in individuals with MRKH identified *ESR1* and *OXTR1* as differentially expressed compared to tissue in controls. *ESR1* was overexpressed whereas *OXTR* was lower expressed, suggesting that variations in these genes may mediate anomalies in uterine development. However, targeted sequencing of both genes in a cohort of 93 patients with MRKH did not identify any convincing variants in the coding sequence of the gene. Two variants were

identified in the 3' or 5' untranslated regions, possibly resulting in microRNA binding alteration or modification in gene expression (248).

TP63

Involvement of *TP63* in Müllerian duct anomalies was considered given the role of *TP63* in the differentiation of the vaginal epithelium in mice. Mice knockout also present cloacal septation defects and anomalies in the development of the genital tubercule (249). Moreover, *TP63* was shown to be regulated by *EMX2*, a gene previously involved in Müllerian anomalies. Reduced expression of *TP63* was found *in vitro* in *EMX2* mutant cells (229). In human, *TP63*-related disorders comprise different overlapping phenotypes with varying combinations of ectodermal dysplasia, cleft lip/palate, split/hand foot malformations, lacrimal duct obstruction, hypopigmentation, hypoplastic breasts and hypospadias (250). Uterine agenesis was described in one patient (251). Targeted sequencing of the gene in a cohort of 200 individuals with uterine malformation identified one heterozygous variant in the 3'untranslated region (c.*374 G > A). By creating a new binding site for two microRNA, the variant was shown to influence *TP63* expression *in vitro* (252).

PBX1

PBX1 encodes a TALE-homeodomain transcription factor that forms a complex with other proteins from the TALE-homeodomain family and Hox proteins to regulate organ patterning during embryogenesis (253). Heterozygous loss-of-function variants have been identified in individuals with congenital anomalies of the kidney and urinary tract syndrome with or without hearing loss, abnormal ears, or developmental delay (named the CAKUTHED syndrome) (253,254). Heterozygous missense variants are less frequently associated with CAKUT but most commonly with genital anomalies (including 46,XY sex reversal). Although no Müllerian duct develops in knockout female mice embryo, only one 46,XX individual with a missense variant in the gene was described with uterine malformation (255). No variant in *PBX1* were identified in cohorts of individuals with MRKH/CUA through targeted sequencing or WES (131,180,256).

Candidate genes suggested by WES studies and CNV analysis

Other candidate genes have been suggested from analyses using microarray, or more recently optical genome mapping, in individuals with MRKH (142,143). A duplication

including *LRP10* (an inhibitor of the canonical Wnt pathway) and *MMP14* (a matrix metalloproteinase) was identified in one affected twin of a monozygotic pair, with differences across tissues suggesting somatic mosaicism (120). Subsequent analysis of these genes in whole exome sequencing data of cohorts of individuals with MRKH syndrome identified five missense *LRP10* variants (with two of them in the same individual) and two variants in *MMP14* (131). The role of these genes in Müllerian duct development still needs to be investigated.

In a series of 30 individuals with MRKH syndrome, intragenic partial duplications of *SHOX* (short stature homeobox), spanning 7–300 kb, were identified in four unrelated individuals with isolated MRKH. In one familial case, the duplication was also found in an affected sister and healthy father (257). Duplication of a SHOX enhancer was reported in another affected individual (258). These results were somewhat controverted by the absence of any intragenic duplication in *SHOX* identified in a larger cohort of 101 individuals with MRKH (259). Furthermore, intragenic duplications have been reported in individuals with idiopathic short stature and Leri-Weill dyschondrosteosis without mention of any uterine anomaly (260). Presence of point mutations in the gene was interrogated in one cohort of 111 individuals by WES, identifying two rare heterozygous missense variants (131). *SHOX*, located in Xp22.33 in the pseudoautosomal region of chromosome X, belongs to the paired homeobox family. The gene does not have any equivalent in rodents and whereas *SHOXb* transcript is expressed in the fetal kidneys (261), expression in fetal Müllerian ducts has not been proven. Whether *SHOX* plays a role in uterine development and whether intragenic duplications of *SHOX* truly confer a risk for uterine malformations remains to be determined.

The region 1q21.1, which includes the gene *RBM8A*, is another locus for which recurrent copy number variants have been identified in individuals with MRKH. *RBM8A* deletion in the presence of some low-frequency noncoding polymorphisms in *RBM8A* results in TAR (thrombocytopenia-absent radius) syndrome. Five individuals with TAR syndrome also had MRKH, one of whom was documented as having a 1q21.1 deletion (129,262–265). Tewes et al.[2015] analyzed the gene *RBM8A* in 167 individuals with MRKH or other Müllerian duct anomalies (266). They identified the TAR-associated SNPs (rs139428292; c.-21G>A) and (rs201779890; c.67+32G>C) in the heterozygous state in 7.2% and 0.6% of their cohort, respectively. However, the association was not statistically significant considering the minor allele frequencies in the non-Finnish European population in GnomAD (2.8% and 2.23%, respectively). Moreover, one rare missense and one rare intronic variant (rs201860373;c*25C>G) were identified in one individual with

MRKH type 2 and two individuals with MRKH type 1, respectively. To date, there is no strong evidence implicating the gene *RBM8A* in the pathogenesis of uterine malformation, and although *RBM8A* could be a candidate gene, wider genetic analyses of affected populations would be necessary to determine the causality of variants in this gene.

Deletions and duplications in 22q11.2, both proximal and distal, are associated with a wide range of anomalies. MRKH anomaly, isolated or in association with other symptoms, can be part of the phenotype (125,128,140,141,267–270). Functional modeling in zebrafish and mice supported haploinsufficiency of *Crkl* as the main genetic driver for CAKUT in 22q11 deletions, with also contribution of *Snap29* and *Aifm3* (271). The causal gene(s) within these regions that are involved in Müllerian development remain to be determined.

Finally, a few recent studies reported novel candidate genes based on exome data analysis of individuals with MRKH or Müllerian anomalies either with a hypothesis-free approach, or by targeting genes previously involved in CAKUT. Only a few genes (*MYCBP2* encoding an E3 ubiquitin-protein ligase; *TBC1D1* encoding a Rab-GTPase-activating protein; *CHD1L* encoding the chromodomain helicase DNA-binding protein 1-like protein; *BMPR1B* encoding a BMP receptor; *DLG5* encoding a membrane-associated guanylate kinase, shown to be involved in ciliogenesis) showed recurrence of rare variants in either two (*MYCBP2, BMPR1B, TBC1D1, DLG5*) and four (*CHD1L*) unrelated individuals each. Segregation analysis was not always performed and there was no functional validation for the missense variants. Heterozygous variants in *TBC1D1*, *CHD1L,*and *DLG5* have been previously identified in individuals with non-syndromic CAKUT (272–275) or syndromes associated with renal anomalies (276). The biological significance of these alterations in relation to CUA still needs further validation.

Syndromes with uterine malformation

Following the observation of recurrent uterine malformations in RCAD syndrome, HFG syndrome, and AARR syndrome, sequencing of the causative genes, *HNF1B*, *HOXA13*, *WNT7A*, respectively, was undertaken in individuals with isolated uterine malformations/MRKH syndrome, with to date, no gene showing major contribution (as reviewed above). Uterine agenesis and uterine malformations have been reported in other Mendelian syndromes or associations, and these other genes might be worth examining in cohorts with isolated uterine anomalies. For example, uterine malformations are frequently reported in Fraser syndrome. Mild bi-allelic mutations in Fraser syndrome

genes (*FRAS1*, *FREM2*, *GRIP1*, and *FREM1*) were identified in individuals with isolated CAKUT (277) and underlined the possibility of mild and incomplete Fraser syndrome phenotypes associated with these genes. Heterozygous variants were reported in one individual with MRKH type 2 (278). Other candidates might be members of the Wnt signaling pathway such as *LRP4*, *PORCN*, and *WNT3*. Considering ciliopathies as one pathological group with different overlapping phenotypes, uterine malformations emerge as a recurrent phenotypic feature, which is not surprising given the role of the cilia in transducing developmental signals—in particular, of the Wnt pathway (279) .

On the other hand, in individuals diagnosed with "isolated MRKH/CUA", exhaustive phenotyping and review of the family history might allow to detect milder signs featuring syndromic presentations. For instance, the presence of short halluces/thumbs or hypothyroidism may target the genetic testing to investigate *HOXA13* or *PAX8* respectively. Based on the syndromic conditions associated with uterine malformation (reviewed in Supplemental data Table 3), the clinical, radiological and biological signs which may be worth to look at in patients with "isolated MRKH/CUA" are summarized in BOX4.

BOX4: Genetic workup suggested in patients with MRKH syndrome/uterine malformation

A. SYNDROMIC CAUSE?

- **Personal/family history**

> Hearing loss (*GATA3*, *GREB1L*)
> Hypermetropia, nanophtalmos (*MYRF*)
> Diabetes MODY (*HNF1B*)
> Renal cyst/CAKUT (*HNF1B*) ; renal agenesis (*GREB1L, HNF1B*)
> Developmental delay/ID/multiple malformation (refer to supplemental data table 3)

- **Clinical exam**

> Hands and feet examination, especially in case of bicorporeal uterus: short, medially deviated halluces and short, proximally-placed thumbs with hypoplastic thenar eminences, distal phalangeal hypoplasia of the first digit (*HOXA13*)

- **Biology**

> Endrocinological workup: LH, FSH, AMH (primary hypogonadism in *MYRF*, uterine hypoplasia in gonadal dysgenesis and in syndromes with hypogonadotropic hypogonadism); androgen (hyperandrogenism: *WNT4*)
> PTH and calcium (*GATA3*)
> Magnesium, TGO-TGP, Hb1Ac and fasting glycemia (*HNF1B*)
> TSH-T4 (*PAX8*)

- **Imagery**

> Spine X-rays: sacral agenesis (Currarino), cervicothoracic vertebral defects (MURCS), vertebral defects (VACTERL)
> Renal ultrasound: renal agenesis, renal ectopia, and other CAKUT
> Hands and feet x-rays (if suspicion *HOXA13* based on clinical exam): fusion or delayed ossification of the wrist bones.

B. GENETIC TESTING:

1) Molecular karyotype (yield: +/-10%)
2) Specific gene test if suspicion of a specific syndromic condition[1]
3) In the absence of syndromic suspicion, consider gene panel testing in the following situations (low yield:5-10%):

- MRKH/ uterine agenesis/hemi-uterus
  + personal or familial CAKUT/costovertebral malformations
  + genetic counselling for recurrence risk in offsprings/siblings
  → *HNF1B, GREB1L, WNT4, PAX8, TBX6*

- Bicorporeal uterus
  + family history of CAKUT/CUA and/or costovertebral malformation:
  → *HNF1B, GREB1L, TBX6*

[1]Consider the HNF1B score established by Faguer et al.(280) for *HNF1B* testing
* *TBX6* and *PAX8* are currently not included in the CAKUT gene panels available in Belgium

3. POSSIBLE EXPLANATION FOR THE LOW DIAGNOSTIC YIELD

Large series of individuals have been sequenced for many genes with a relatively low rate of compelling genetic variation (Supplemental data Table 2). The difficulty in identifying genetic causes for uterine malformations may have several explanations. First, our current understanding of genes and pathways involved in uterine development is partial, and animal models cannot always be used as a reference since uterine development is specific in humans, especially when studying fusion and absorption septal defects (9).

Second, uterine malformations may be highly genetically heterogeneous in their etiology, with each single gene explaining only a few cases, as in CAKUT, for instance, in which about 60 genes are involved in monogenic non syndromic, or mildly syndromic CAKUT (198). Alternatively, these anomalies may be shown to be oligogenic/polygenic in origin, such that a combination of variants in the same regulating pathway or epistatic effects of several rare variants and polymorphisms might be required for expression of the phenotype (183). Identifying such polygenic factors is particularly challenging in rare diseases, as association studies need large cohorts to achieve statistical significance, making studies on uterine malformations susceptible to being underpowered. In cases with a monogenic cause, additional factors may be needed for full penetrance, such as combination of hypomorphic common variant alleles in coding or non-coding region in *trans* with rare variants in the same gene (e.g., as seen for *TBX6* and spondylocostal phenotypes) (218). There is a risk of overlooking causal variants when found in asymptomatic individuals and incomplete penetrance must therefore be considered in the interpretation of sequencing data.

Third, the role of somatic mosaicism for *de novo* mutations may be suggested by discordance in monozygotic twins and the absence of recurrence in the offspring of affected women (which could be explained theoretically by lethality in germline). Among five pairs of discordant MZ (monozygotic) twins, differences in copy number variants across tissues were demonstrated and included duplication of *LRP10*, an inhibitor of the canonical Wnt pathway, and of *MMP14*, a matrix metalloproteinase that might play a role in Müllerian ducts cell migration (120).

Finally, besides genetic factors, there is evidence of environmental factors contribution to uterine malformations. In humans, the best-known example is the association of uterine anatomical deviations (i.e., small endometrial cavities, T-shaped uteri or dilated cornuate areas) with prenatal exposure to DES (diethylstilbestrol), a synthetic estrogen widely used to treat abortion risk from 1940 to 1970. The frequency

and extent of malformations depends on the starting date of fetal exposure, the earlier the exposure, the greater the effect (4). DES is considered as an endocrine-disrupting chemical (EDC) and, like other chemicals such as bisphenol A, phthalates or pesticides, it has been proven to interfere with endocrine systems in mammals (281). Binding to estrogen nuclear receptors and altering regulatory regions of developmental genes by epigenetic modifications, EDCs may regulate transcription of genes essential for uterus development. Exposure during a critical period of development may disturb normal genital tract formation (282) (Figure 12). In mice, exposure to DES has been shown to shift the pattern of expression of *Hoxa9-11* to a more caudal distribution and to induce an anterior transformation of the reproductive tract. *HOXA10* expression is first increased by the agonist action of DES, mediated by the ERalpha. Longer *in utero* exposure will then result in persistent aberrant methylation (22). In third-generation DES children (children of women/men exposed to DES in utero), an increased incidence of hypospadias has been demonstrated in sons, supporting an epigenetic alteration occurring during reprogramming of the primordial germ cells in their parents, or alternatively transgenerational transmission of epigenetic factors. Hypermethylation of the *HOXA* family was suggested as a compelling hypothesis to explain the defect (4). In regards to genital tract malformation in girls, a recent study reported 12 individuals with uterine malformation/759 third- generation granddaughters (including 3 MRKH patients, 6 with bicornuate uterus and 3 with a "doubling" uterus). Given the observation of higher frequency for MRKH syndrome compared to the general population (20x higher than expected), the authors raised the question of a possible link with DES exposure through epigenetic effects (283). However, they specified that no definitive conclusions should be drawn given the limited number of patients and the possibility of recruitment bias. Moreover, previous studies on large cohorts of third generation grand-daughters did not report higher frequency for uterine malformations, suggesting that epigenetic alteration of a MRKH gene is rather unlikely (4,283).

It is currently unknown whether other EDCs may cause uterine malformations in the range of doses to which human fetuses might be exposed. In mice, *Hoxa10* transcription was altered following bisphenol A exposure (21,284), and epigenetic modifications of the *Hoxa10* promoter were noted (285). In rats, exposure to high dose of phthalates (520 mg/kg/ day mixture of five different phthalates between the 8th to the 19th day of gestation) has been associated with uterine malformations (i.e., uterus agenesis, hemi-uterus, absence of vaginal opening) and was suggested as a promising animal model to study pathogenesis of MRKH syndrome (286).

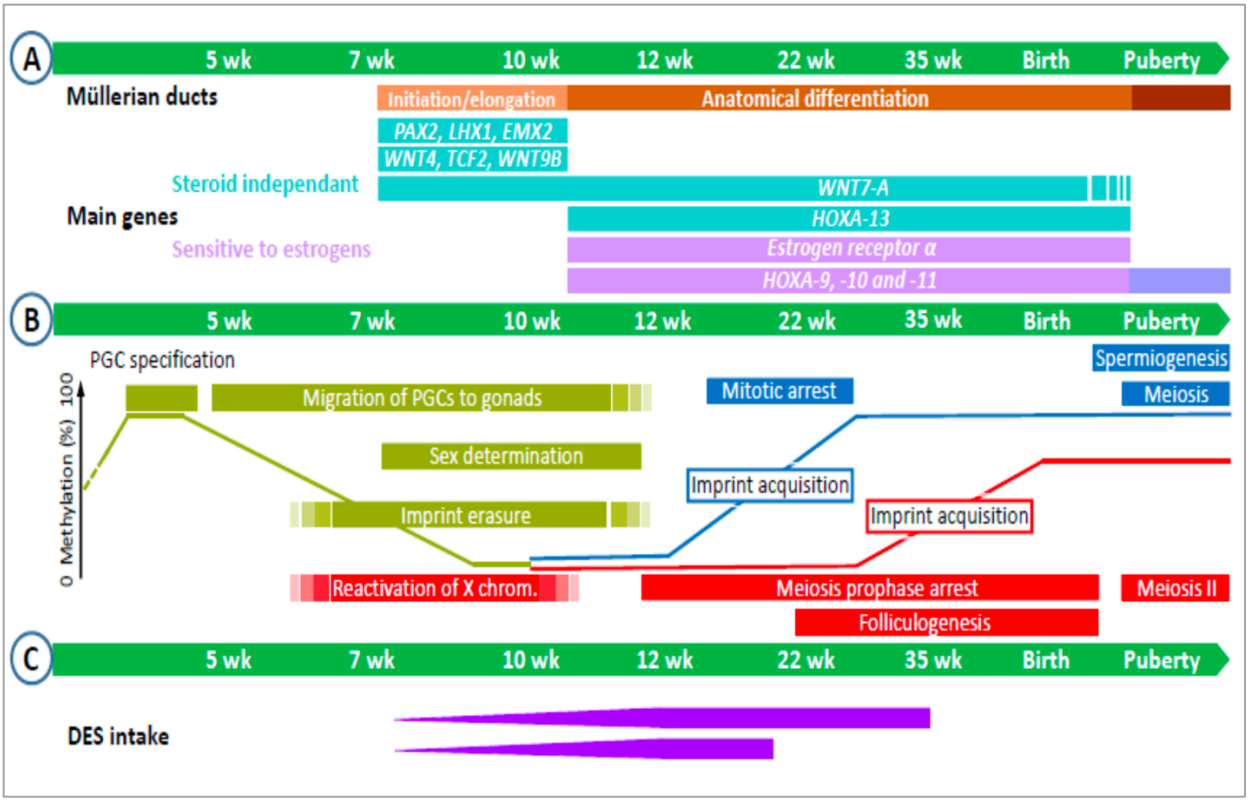


Figure 12. Impact of DES intake on Müllerian ducts development.
Period of DES intake (C) in parallel with the Müllerian ducts developmental steps (A) and the Primordial Germ Cells specification and reprogramming (B). Deregulation of the hox programs from 10 weeks onwards is the hypothesis to explain abnormal T-shaped uterus in second generation exposed daughter. Germ cells are exposed to DES during the reprogramming program which may result in epigenetic alteration of developmental genes in the third-generation DES children. The time of pregnancy is indicated non linearly in weeks from the first day of last menstrual period. (From Guerrier 2022(4)).

Epigenetic alterations of developmental genes, secondary to environmental factors or genetic causes, can be potentially limited to one organ or tissue. Epigenetic causes could also explain sporadic cases of uterine malformations and MRKH syndrome as well as discrepancy observed between monozygotic twins. Comparison of transcription and methylation profiles in post-surgery uterus myometrium from adult individuals with MRKH and controls highlighted differences in both transcription and methylation for six genes potentially relevant to the development of the female reproductive tract (*CDH5, MFAP5, WISP2, HOXA5, PEG10, HOXA9*) (287). However, further studies are necessary to determine links between epigenetic modifications of developmental genes and uterine development as well as how environmental factors are involved in these processes.

IV. INTERPRETATION OF WHOLE EXOME SEQUENCING DATA AND *IN VIVO* MODELLING OF CANDIDATE GENES

Whole exome sequencing (WES) enables the sequencing of the coding regions of approximately 20,000 protein-coding genes of an individual's genome. Comparing the data to a reference genome generates a list of variants (single nucleotide variations and small indels). Since many of these variants are benign, data analysis focuses on narrowing down the list to those more likely to be significant. Common filters include allele frequency (e.g., selecting variants with a minor allele frequency <1%) and coding impact (such as non-synonymous variants or those predicted *in silico* to affect protein function).

For genes already associated with the disease, the pathogenicity of a variant can be assessed using the ACMG/AMP guidelines, an internationally recognized framework for evaluating gene variant pathogenicity (288). One of the criteria involves phenotype correlation, and reverse phenotyping can sometimes provide additional evidence of pathogenicity by identifying overlooked clinical features. The ACMG/AMP guidelines and the approach of reverse phenotyping are discussed in the first part of this section.

In cases where no causal variant is identified in known genes, exome analysis can help to identify compelling variants in potential novel candidate genes. For these variants—those that are rare in the general population and predicted to be deleterious—key criteria for evaluating gene relevance include the gene biological function (e.g., interactions with disease-related genes or pathways), expression data (such as expression during embryonic development or in relevant tissues), and evidence from animal model demonstrating involvement in embryonic development. Constraint metrics can also indicate whether a gene is intolerant to heterozygous loss-of-function variants, suggesting a higher likelihood of developmental disease in the presence of such variations. For new candidate genes, the existing literature may not yet provide sufficient evidence for causality in regards to uterine malformation, and studies using animal models may be needed to gather additional support.

Although mice are the primary animal model for studying female genital tract and kidney development, zebrafish offer several advantages: they are less expensive, highly fecund (producing 50–200 ex utero transparent embryos per pairing), have rapid development (reaching adulthood in three months), and are easily modified genetically. Zebrafish do not have Müllerian ducts, and it is uncertain how conserved is the development of their gonadal duct compared to the Müllerian ducts (BOX5). However, zebrafish have proven their utility in studying kidney development and renal disease, their embryonic kidney (pronephros) being a simplified and conserved model of the

mammalian nephron. The second part of this section introduces CAKUT modeling in zebrafish.

1. INTERPRETATION OF SEQUENCE VARIANTS IN KNOWN CAUSATIVE GENES

The ACMG/AMP classification

The American College of Medical Genetics and Genomics (ACMG) guidelines [2015], provided standards for the interpretation of sequence variants in known causative genes associated with Mendelian disorders, which are today commonly used in diagnostic labs (288). The process combines population data, computational and predictive analysis, functional criteria and allelic and co-segregation data to classify variants in one of five categories, ranging from variants of benign (or likely benign) clinical significance to those that are pathogenic (or likely pathogenic). Variants of unknown significance include all the variants in between, that do not meet the criteria to be classified as likely benign or likely pathogenic. Since then, the Sequence variant interpretation working group (Clingen) has provided additional general guidance to use the ACMG criteria for the purpose of improving consistency and uniformity in variant interpretation (289–293). The probability that likely pathogenic and pathogenic variants are truly pathogenic reaches 0.9 and 0.99 respectively. The classification system does not exclude Mendelian genetic conditions with decreased penetrance, but recommend, in these cases, to include information about decreased penetrance and variable expressivity when reporting genetic results. When assessing segregation data (PP1 criterion) for autosomal dominant conditions with reduced penetrance, only affected relatives should be considered (289).

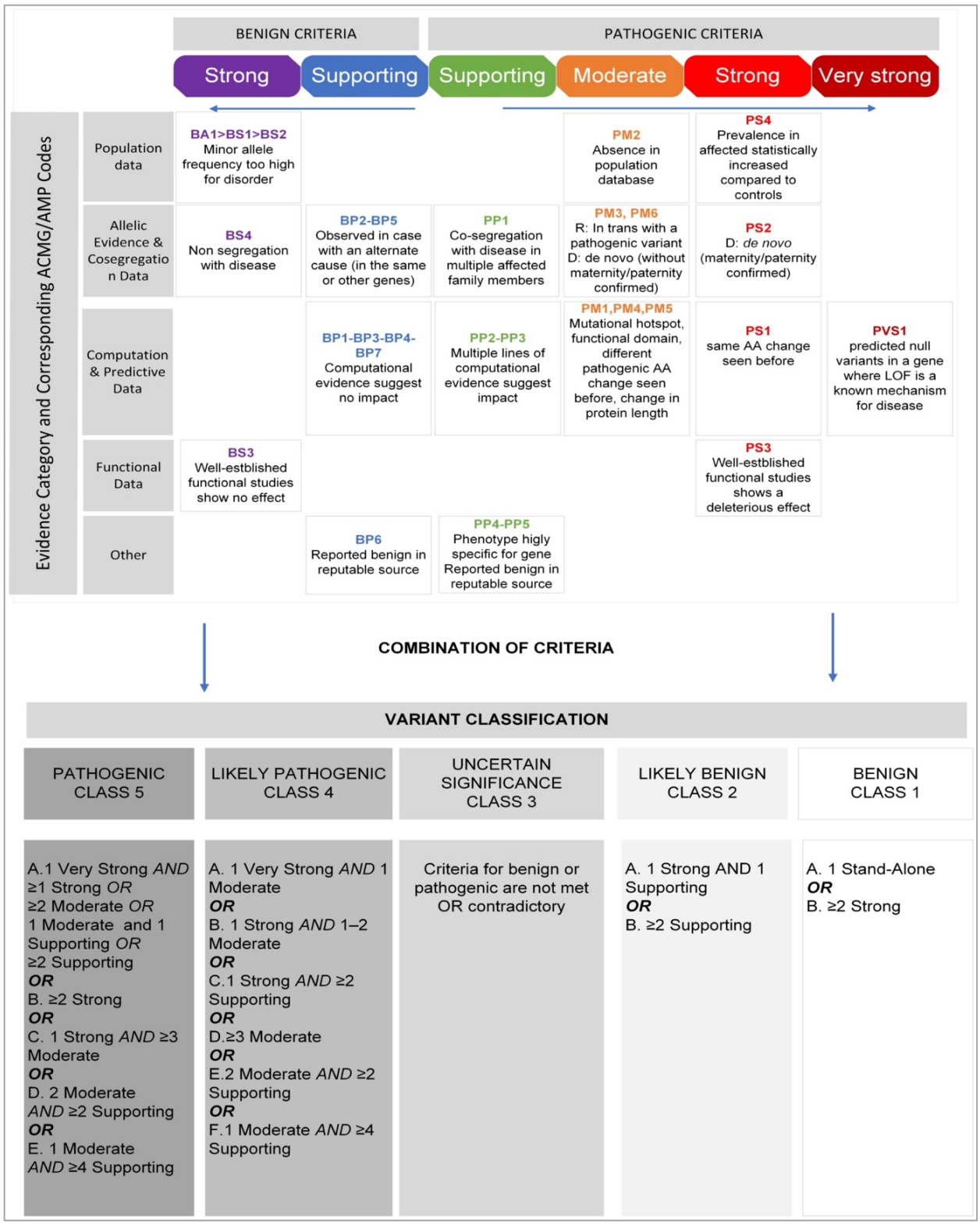


Figure 13. Sequence variants are classified based on a combination of criteria.
Evidence criteria are divided into those that support a benign (B) or pathogenic (P) effect (first letter of the code) and given their relative strength (second letter(s) of the code: VS= Very strong, S= strong, M= moderate; P= supporting; A=stand-alone). One criteria can be upgraded to higher strength when more evidence are available. R= recessive; D= dominant ((288,294).

Reverse phenotyping

Reverse phenotyping involves evaluating specific clinical features after a genetic diagnosis is suggested by sequencing results. This approach has been shown to strengthen the evidence for the pathogenicity of variants identified through WES. For instance, in individuals initially diagnosed with isolated CAKUT who carry a potentially causal variant, further clinical examination helped confirm disease causation in 8 out of 85 cases, while in 23 out of 85 cases, the variant was considered unlikely to be causal (295).

According to the ACMG/AMP guidelines and their updated version (296), the PP4 criterion assesses phenotype specificity and may be applied when a particular constellation of clinical features aligns with molecular findings. In cases of genetic heterogeneity for the phenotype, applying this criterion requires that other potential genetic causes have also been analyzed with the genetic test performed. This criterion can be upgraded to moderate evidence if very specific phenotypic features are present, and to strong evidence if additional findings—such as an enzymatic activity assay, specific blood markers, a methylation signature, muscle biopsy analysis, or a drug response—are pathognomonic for a particular genetic cause (296).

In the initial clinical evaluation of individuals with uterine malformations, mild extrauterine features may have been unexamined. Based on molecular results, further clinical, biological, or radiological assessments may reveal distinct clinical features associated with syndromic or minimally syndromic conditions (e.g., caudal pancreas agenesis and renal cysts with an *HNF1B* variant; hypoparathyroidism or hearing loss with a *GATA3* variant; subclinical hypothyroidism with a *PAX8* variant). Additional features worth investigating, depending on the identified variant, are described in BOX4 and Supplemental Table 3.

2. VALIDATION OF CANDIDATE GENE IN ZEBRAFISH

For genes not previously implicated in a disease, evidence for a role of the candidate gene in the disease should be demonstrated, and animal models have been the *in vivo* favorite models so far. Despite its simple architecture, the pronephros of zebrafish shares a conserved structure and functional similarities with the mammalian nephrons, providing an attractive model to study the genes and pathways involved in vertebrate kidney disease and nephrogenesis.

The zebrafish kidney

The zebrafish embryonic kidney (pronephros) consists of a pair of segmented tubules linked in their proximal end to a fused single glomerule, and connecting in their distal part to the cloaca. The pronephros arises 12 hours post fertilization (hpf) from bilateral stripes of renal progenitors in the intermediate mesoderm, and both tubules are formed at 24 hpf. The glomerule primordia fuse at 36-40hpf, and filtration starts between 40-48hpf. Mesonephrogenesis starts around day 10-12 to form mesonephrons which connect with the distal pronephric tubules to form the adult kidney (mesonephros) (Figure 14.A). In zebrafish, the total number of nephrons (around 150-300) correlates with the body weight and neonephrogenesis goes on during all the adult life (297). Unlike mammals, zebrafish do not develop a metanephros. However, the molecular pathways involved in intermediate mesoderm specification and nephrogenesis are conserved, allowing for meaningful comparisons between different kidney forms despite differences in spatial organization and complexity. Key transcription factors such as Pax2a, Pax8, Lhx1a, and Osr1 play crucial roles in specifying renal progenitor fate within the intermediate mesoderm in both zebrafish and mammals (298–300). The nephric duct functions as a terminal inducer of mesonephric and metanephric mesenchyme (301). Nephrons, the basic functional units of all vertebrate kidneys, exhibit similarities in their developmental processes and show remarkable conservation in the structure and function of their segments. These segments are composed of distinct epithelial cell types, each with specialized roles. Furthermore, many genes expressed in specific segments of mammalian nephrons are also found in corresponding segments of the zebrafish pronephros (Figure 14.B).

Studies in zebrafish have provided insights on how nephrons differentiate into segments and the pathways involved, unveiling an important role for retinoic acid signaling (Figure 15). In *aldh2* mutants (zebrafish embryo deficient for RA biosynthesis), or after chemical treatment with diethylaminobenzaldehyde (DEAB) (chemical that blocks Aldh enzymatic activity), embryos fail to form the glomerule and proximal segments of the pronephros. Conversely, exposure to exogenous RA treatment between the end of gastrulation and early somitogenesis induce expansion of the proximal segments and reduction of the distal segments. Alterations are already visible at the 8 somites stages when the renal progenitor cell fields are subdivided into rostral and caudal domains based on their gene expression pattern, showing that the RA gradient acts very early to promote the proximal, and maybe inhibit the distal fates (297,302). In mammals, retinoic acid signaling was also shown to be important for the primary ureteric bud outgrowth (through

regulation of Ret expression), for branching morphogenesis and for proper insertion of the nephric ducts in the cloaca (43,303). Finally, studies in zebrafish have contributed to understand the dynamic processes (mesenchymal to epithelial transition, cell migration, stretch-activated proliferation) that determine the final structural arrangement of the tubules (304).

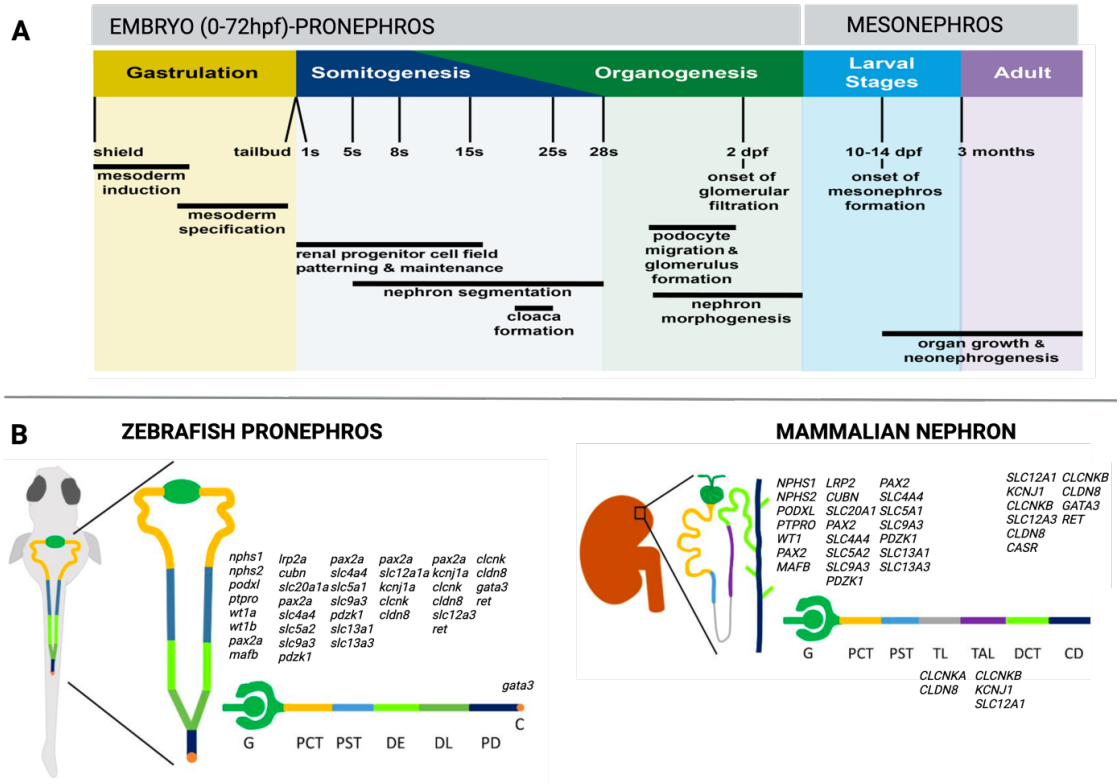


Figure 14. The renal system in zebrafish.
A. Developmental stages of the renal systems in zebrafish (from Gerlach 2013 (297))
B. Comparison of nephron segment patterning between the zebrafish and mammalian metanephros. Specificity in gene expression of proximal and distal segments is conserved between zebrafish and mammals (from Elmonem 2018 (305)). Zebrafish lacks an equivalent of the loop of Henle which facilitates urine concentration in mammals. G= glomerule; PCT= proximal convoluted tubule; PST= proximal straight tubule; DE= distal early; DL= distal late; CD= collecting duct; TL= thin limb; TAL= thick ascending limb; DCT= distal convoluted tubule; PD= pronephric ducts; C= cloaca

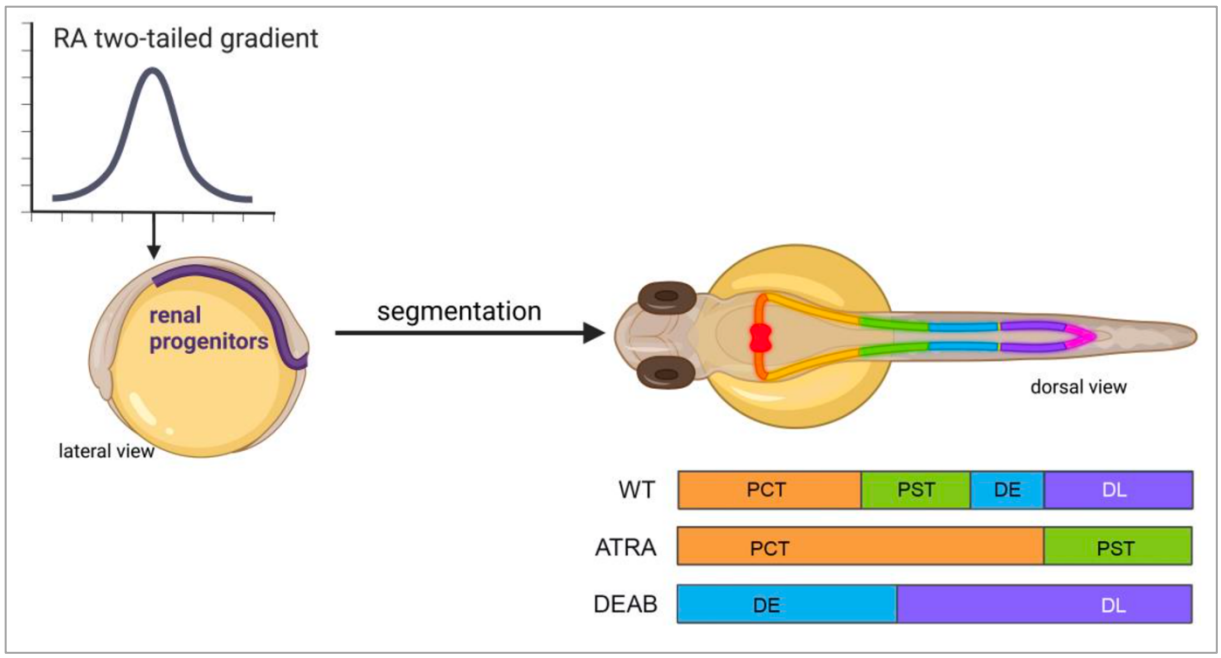


Figure 15. Retinoic acid and patterning of the pronephros in zebrafish.
Patterning of the proximo-distal nephron segment identities is mediated by retinoic acid (from Hawkins 2023 (306)). PCT= proximal convoluted tubule; PST= proximal straight tubule; DE= distal early; DL= distal late; ATRA = RA agonist; DEAB= RA antagonist.

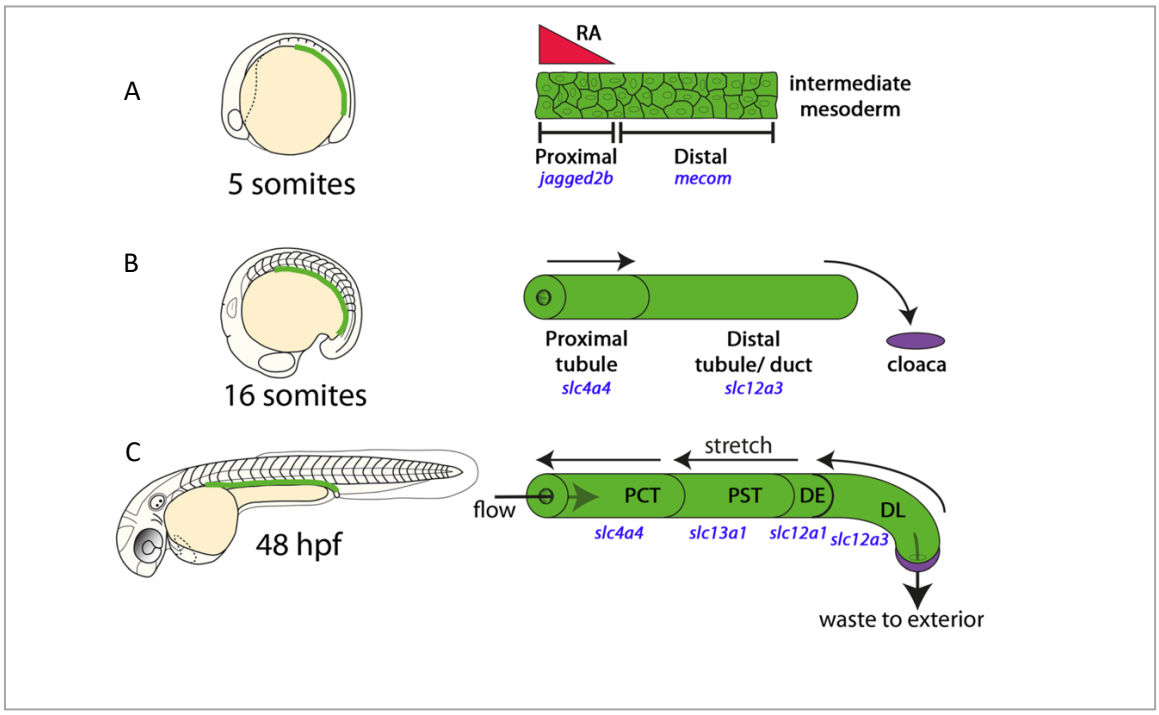


Figure 16. The dynamic processes in pronephric tubules formation.
A. Formation and patterning of the intermediate mesoderm. The PCT, PST and DE form from intermediate mesoderm exposed to RA while the DL segment forms from the IM not exposed to RA.
B. Formation of a tubular epithelium by processes of mesenchymal-to-epithelial transition and cell-to-cell adhesion. By 20 somites, a lumen and markers of apical-basal polarity are present. At that time, a caudal shift in the proximal-distal domains is observed. A short medial elongation of the tubules is necessary to reach the cloaca which then fuse (22 somites stage (20hpf)). Migration of distal fate cells and compaction of the distal late segment result in strectching and elongation of rostral segments (PCT,PST and DE). The Bmp signaling and Wnt (both PCP and canonical) are involved in cloaca formation.
C. Sarting at 29hpf, a rostral flow-induced collective cell migration induce circumvolution of the proximal circumvulated tubules and stretches of the intermediate and distal early segments. Obstructing the tubules, decreasing the glomerular filtration or inhibiting motile cilia formation all cause inhibition of the rostral migration (From Naylor and Davidson 2017 (304)).

BOX 5. Is the ovarian duct in zebrafish a homologue structure of the Müllerian ducts in mammals?

Detailed description of the development of the gonadal duct is limited in zebrafish, but some description exists in the medaka, another teleost. In medaka, initiation of gonadal duct formation was first identified 20-30 days after hatching. Epithelial cells of the gonadal duct originates from the coelomic epithelium similar to the Müllerian duct cells in mammals (14,307). Studies on molecular pathways involved in the ovarian duct formation and elongation in zebrafish are rare. Recently, *wnt4a* loss-of-function was shown to cause partial agenesis of the gonadal duct in zebrafish and medaka, showing homologies with the phenotype in mammals and suggesting that both the Müllerian ducts and gonadal duct may share common genetic networks in their development. Further studies are however needed. MicroCTscanner and histology are available methods to analyze the structure of the gonadal ducts in adult zebrafish (308)

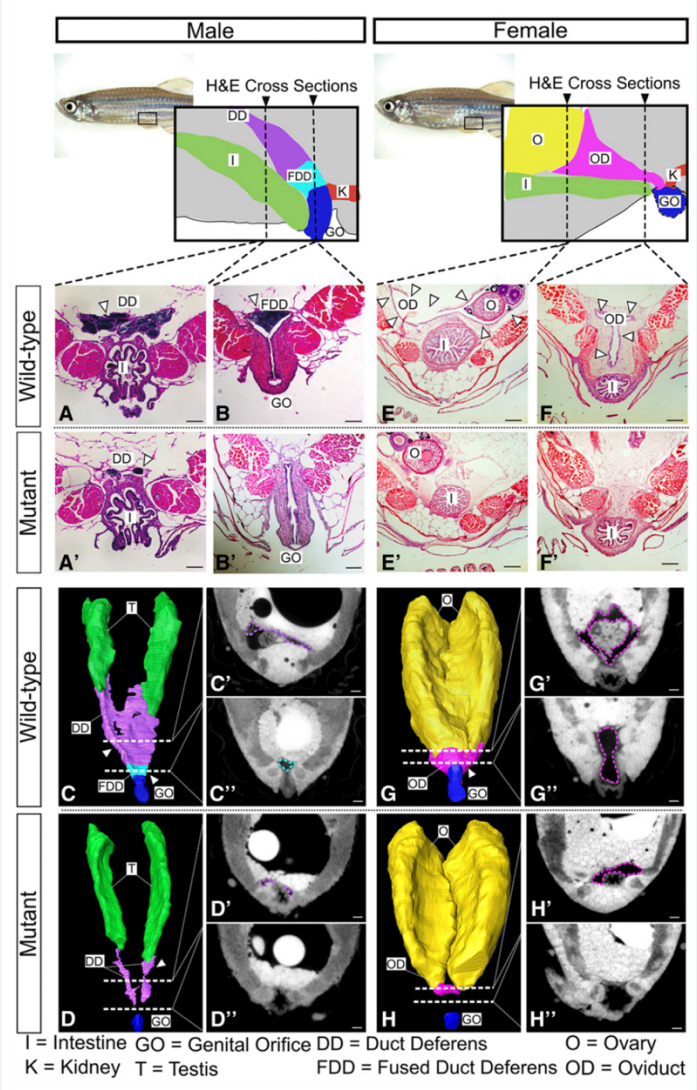


Figure 17. The ovarian duct in *wnt4*$^{-/-}$ zebrafish
Abnormal development of the male and female gonadal duct in *wnt4a*$^{-/-}$ mutants compared to wildtype (from Kossack 2019 (308))

Methods to investigate candidate genes for CAKUT in zebrafish

In order to investigate the phenotype associated with one specific candidate gene[8], several methods exist to knockout or knockdown its expression in zebrafish. Essential prerequisite is the presence of an identifiable zebrafish orthologue (BOX6), which is the case for around 70% of the human genes (309). However, if 47% of them have a one-to-one orthologous relationship (310), 23% have two (or more) zebrafish orthologues secondary to an ancient additional round of duplication of the teleost genome (approximately 350 million years ago). Duplicated genes may have redundant function, or neofunctions. In consequence, identifying the most likely orthologue may sometimes be challenging, and knocking out the duplicated genes might be necessary to display a phenotype (298,310). Morpholino and CRISPR-Cas9 technologies will be described in more details below and are compared in Table 4. Mutants resulting from forward genetic screen[9] also exist for many genes. A vast catalogue of the available mutants and transgenic lines, as well as gene-specific expression data and link to publications, are curated in the ZFIN database (www.zfin.org) (311).

Table 4. Comparison between morpholino and CRISPR-Cas9 approaches (from Elmonem 2018) (305)

	MORPHOLINO	CRISPR-CAS9
Technique first described in zebrafish	Nasevicius and Ekker (2000)	Hwang et al (2013)
Genetic target	mRNA	Genomic DNA
Stage of inducing mutagenesis	1-4 cell stage	1 cell stage
Mutation site	No DNA mutations	Specific DNA sequence
Mutational effect	Deficiency	Deficiency/gain
Difficulty in confirming the mutant genotype	Easy	Easy
Efficiency of mutagenesis	High	High
Mutant model	Transient	Permanent
Time, effort and ressources	+	++
Off-target effects	+++	+

Morpholinos

Morpholinos are synthetic short chain of about 25 oligonucleotides (constituted of a nonribose morpholine backbone) that confer gene knockdown by complementing with their

[8] The genotype-driven approaches are named Reverse genetics. A candidate gene is modified by genetic engineering methods to establish the phenotype associated with mutations disrupting this gene.

[9] Forward genetic consists in phenotype-driven approach. Following induced mutation (for instance by exposure to chemicals such as ENU), mutants are selected based on their phenotype. Then, genomic studies are performed to determine the causal variant.

target RNA. They can either block translation initiation in the cytosol (by targeting the 5' UTR through the first 25-bases of the coding sequence) or can modify pre-mRNA splicing in the nucleus (by targeting splice junctions or splice regulatory sites) (Figure 18.A). The effect of Morpholino is transient with an efficacy limited to 3-5 days. Demonstration of a similar phenotype with multiple MO targeting the same gene is necessary to rule out that the phenotypes observed result from off-target effects (310).

CRISPR-Cas9

The genome editing CRISPR-Cas9 system is derived from components of an adaptative bacterial immune system that allow bacteria to recognize and digest exogenous DNA. Cells are transfected with the endonuclease Cas9 and an engineered guiding RNA (gRNA). The guide RNA includes a target-specific CRISPR RNA (crRNA) and a transactivating crRNA sequence (tracrRNA), necessary to recruit the Cas9 nuclease. For cleavage by Cas9, a specific PAM (protospacer adjacent motif) sequence of three nucleotide (NGG) must be present on the DNA sequence immediately downstream of the 3' target. The nuclease generates double-strand breaks in the DNA, triggering one of several DNA repair system endogenous to cells (the non-homologous end joining DNA repair system and homologous recombination). The predominant NHEJ repair system will generate deletion, duplication, indel that will induce, for some of them, loss-of-function of the gene (knockout model). A template DNA can be transfected with the Crispr-Cas9 system to engage homologous recombination, and generate one specific mutation (knock-in model).

Specificity of the system is guided by RNA sequences and consequently, easy to engineer to generate breaks to virtually any genomic DNA sequence. The system is highly efficient and specific, decreasing the risk of off-target effects (312). Because of its high efficiency, biallelic invalidation of the target genes can be obtained in mosaic in the F0 generation (following injection in the blastocyte) allowing to quickly evaluate the presence of phenotypic consequences (Crispant model). The mosaic larvae can be grown up and paired with wildtype adults. F0 adult carrying mutations in their germline will pass the mutation to the F1 generation. After genotyping, heterozygous are incrossed to analyze the phenotype in the F2 generation (Figure 18.B).

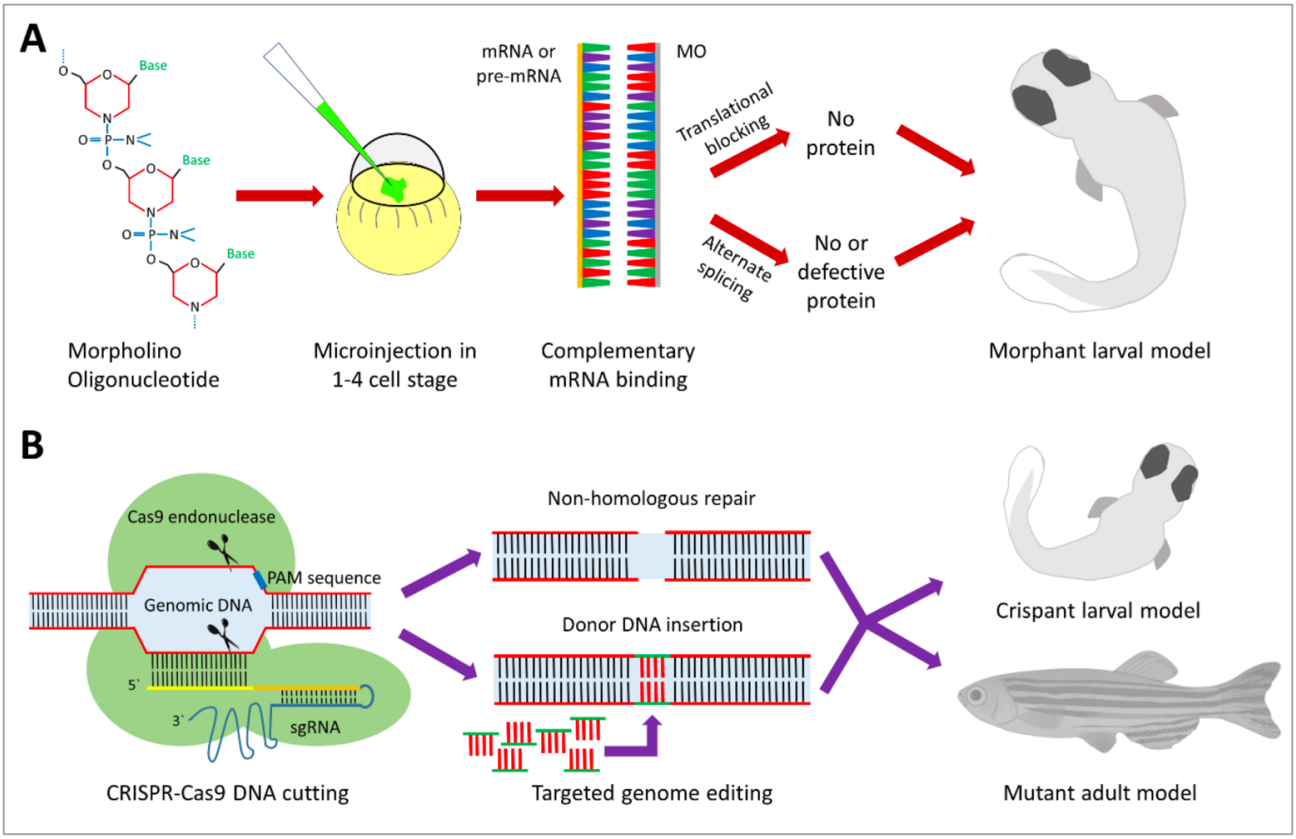


Figure 18. Reverse genetics in zebrafish using morpholinos (A) and CRISPR-Cas9 (B). From Elmonem 2018 (305)

BOX 6. Homologues, orthologues and paralogues

Homologous genes descents from a common ancestral DNA sequence. They are orthologues if they diverged from each other as a consequence of a speciation event, Orthologues are related genes in different species that share high similarities in their DNA sequences, protein domains and functions. *WNT4* in humans, *Wnt4* in mice and *wnt4a* in zebrafish are one example of orthologous genes. One human gene may have several orthologous in zebrafish. Paralogues are related genes within the same species that diverged from each other as a consequence of duplication events. They may share similar functions or become different in sequence and functions over time. For instance, in humans, *HOXA11, HOXC11* and *HOXD11* are paralogous genes, as well as *HOXA9, HOXA10, HOXA11 and HOXA13*

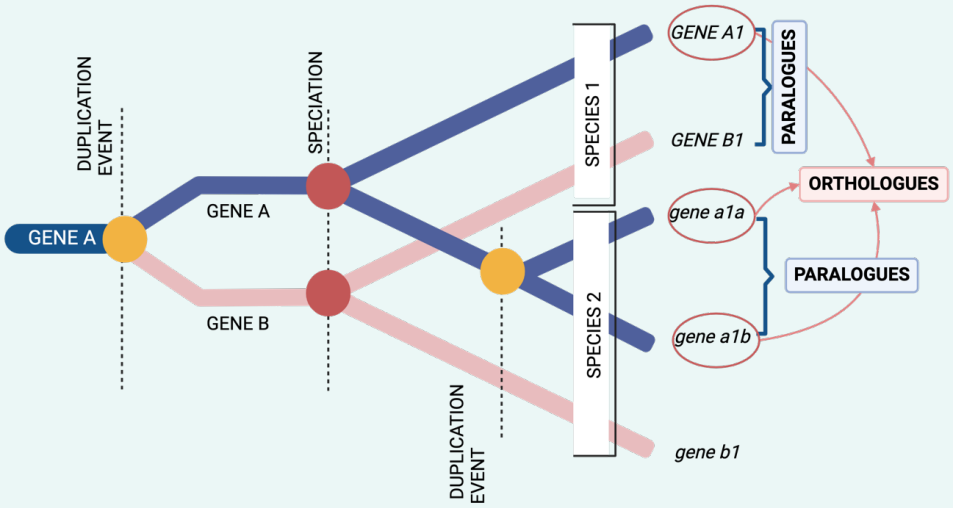


Figure 19. Homologues, orthologues and paralogues.
Paralogues are defined as genes for which the most common ancestor node is a duplication event (yellow node). Orthologues are defined as genes for which the most common ancestor node is a speciation event (red node). Created in BioRender.com

Assessment of the renal phenotype

Although not exclusive to renal disorder, some systemic phenotypes (pericardial or total body edema, pronephric cysts, curved body axis, hydrocephalus) are frequent morphological changes visible under the microscope in zebrafish with pronephros defects. Different methods exist to evaluate further the pronephros/mesonephros morphology and patterning. The methods used during the first investigation of our candidate gene in zebrafish are illustrated in Figure 20.

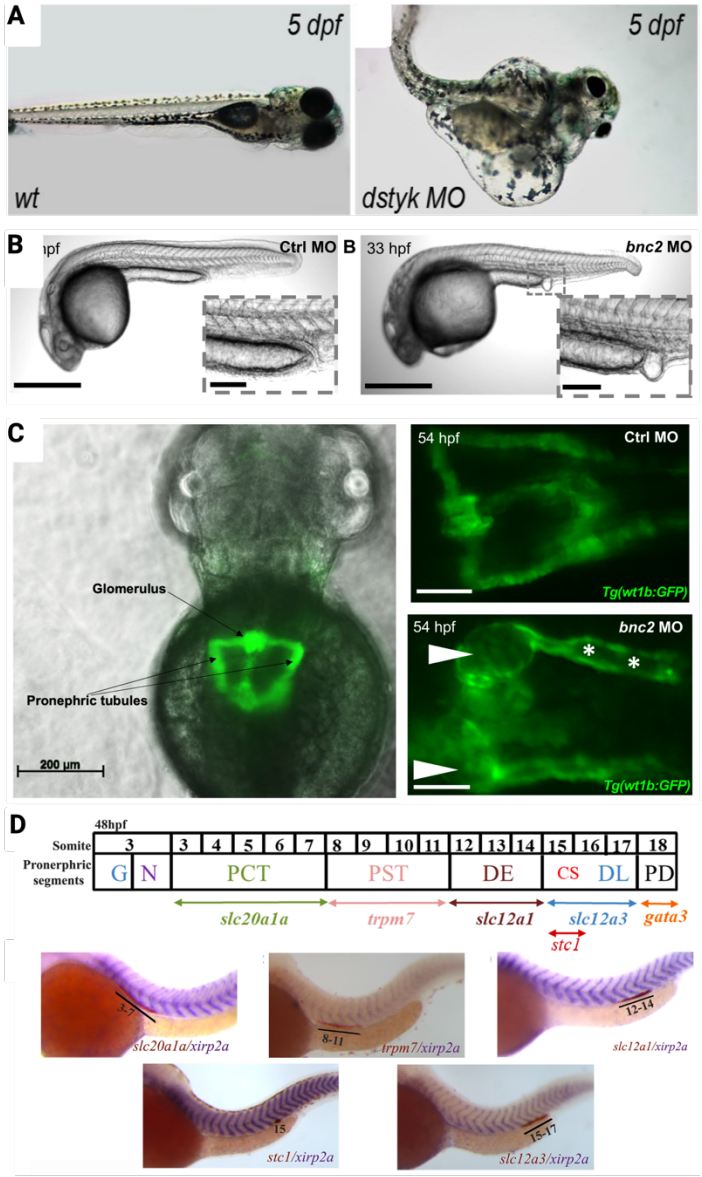


Figure 20. Methods to evaluate zebrafish models of CAKUT
A.Brightfield microscopy allows to screen for morphological defects: comparison of wildtype and *dstyk* morpholino, showing edema and curvature in 5dpf *dstyk* mutants (from Sanna Cherchi, 2013 (313)).
B.Brightfield microscopy allows to visualize the terminal part of the pronephric duct: comparison of wildtype and dilation in *bnc2* morpholino (from Kolvenbach,2023 (314))
C.The transgenic line wt1b::GFP: synthesis of the GFP (green fluorescent protein) where the gene is expressed (in this case wt1b) enables the fluorescent visualization of the glomerules and proximal tubules by fluorescent microscopy. Cyst and dilation of the proximal tubules are visible in *bcn2* embryos (from Outtandy, 2019 and Kolvenbach, 2023) (298,314)
D.Whole-mount in situ hybridizations use labled RNA probes complementary to the mRNA of genes specific to one segment of the pronephros (from Fatma, 2021) (315).

Several genes associated with CAKUT in humans have been investigated in zebrafish, variably showing defective tubular differentiation, abnormal segmentation patterns, pronephric cysts, abnormal tubules (dilated, deformed, convolution defects) or cloacal deformities (Table 5).

Table 5.Characterized embryonic models of CAKUT in zebrafish

Disease	OMIM	Heredity	Gene	Methodology	Phenotype	Reference
Papillorenal syndrome	#120330	AD	*pax2a*	ENU	Abnormal pronephros development, defective tubular differentiation and patterning, abnormal cloaca morphogenesis	(316)
Di George syndrome	#188400	AD	*crkl, aifm3, snap29*	MO, CRISPR	Major convolution defects, reduced length of pronephric tubules	(271)
Denys-Drash syndrome	#194080	AD	*wt1a*	MO	Disruption of glomerular morphogenesis and differentiation	(317)
Renal Cyst and diabetes syndrome	#137920	AD	*hnf1ba/b*	MO, Retroviral insertion	Abnormal nephron segmentation, tubular dysfunction	(318)
Renal hypodysplasia	*604994	AD	*six2*	MO	Abnormal glomerular morphogenesis (altered pattern of wt1 expression)	(319)
***Renal hypodysplasia, Bilateral renal* agenesis**	*112262	AD	*bmp4*	MO	Abnormal glomerular morphogenesis (altered pattern of wt1 expression)	(319)
Renal hypodysplasia/aplasia 3	#617805	AD	*greb1l*	ENU, MO, CRISPR	Dilated tubules, deformed junction between proximal convoluted tubules and the neck, pronephric cysts, pericardial edema, early mortality	(147,154)
Classic bladder exstrophy	* 600366	AD	*isl1*	MO	Abnormal urinary tract development	(320)
CAKUT1	#610805	AD	*dstyk*	MO	Cloacal deformities, growth retardation, pericardial edema, small fins, abnormal jaw development	(313)
Branchiooculofacial syndrome	#113620	AD	*trm* (forward genetic screen, mutation in *tfap2a*)	ENU	Abnormal DE segment differentiation, anomalies in the craniofacial cartilages	(321)
LUTO	#618612	AD	*bnc2*	MO	Pronephric distal outlet obstruction	(322)

Adapted from Elmonem et al., 2018(305). Diseases are in italic, and the OMIM number correspond to the gene OMIM number, when the association is suggested but not confirmed.

V. SUPPLEMENTAL DATA

1. CLASSIFICATION SYSTEMS FOR UTERINE MALFORMATIONS

Supplemental data Table 1. Comparison of the main classification systems for uterine malformations.

	ESHRE/ESGE classification system Grimbizis 2013	***Updated embryological-clinical classification for female genitourinary Acién 2011***	***Vagina, Cervix, Uterus, Adnexae and associated Malformations system (VACUAM system) Oppelt 2005***	***AFS/ASRM classification system American Fertility Society 1988***
Main Classes for uterine malformations	U0, Normal uterus U1, Dysmorphic uterus (a. T-shaped, b. infantilis, c. others) U2, Septate uterus (a. partial, b. complete) U3, Bicorporeal uterus (a. partial, b. complete, c. bicorporeal septate) U4, Hemi-uterus (a. with or b. without a rudimentary cavity) U5, Aplastic uterus (a. with or b. without rudimentary cavity) U6, For still remaining unclassified cases + Include an independent classification system for cervical and vaginal anomalies	1. Unilateral genito-urinary agenesis or hypoplasia (with or without contralateral müllerian agenesis) 2. Uterine duplicity with a blind hemivagina (or atresia) and ipsilateral renal agenesis 3. Isolated or common uterine or utero-vaginal anomalies 3. A. Any of the ASRM-classified müllerian ducts anomalies based on ASRM classification 3. B. Müllerian tubercle anomalies 3. C. Both müllerian ducts and tubercle anomalies 4. Accessory uterine masses and other gubernaculum dysfunctions 5. Anomalies of the urogenital sinus 6. Malformative combinations	(U)0 Normal (U)1 Arcuate (1a)-Septate (1b-1c) (U)2 Bicornate (U)3 Hypoplastic uterus (U)4 rudimentary or aplastic unilaterally(4a),bilaterally (4b) (U)# Unknown + Include an independent classification for vagina (V), cervix (C),and adnexa (A).	Class I, Aplasia, hypoplasia Class II, Unicornuate Class III, Didelphys Class IV, Bicornuate Class V, Septate Class VI, Arcuate Class VII, DES-related anomaly
Renal anomalies	Reported in the addendum « associated non-Müllerian anomalies »	Included in the classification system	Reported by the letter (R) if present	Not included in the classification system
Advantages	Systematic and precise reflection of the entire malformation	Accounts for frequently associated anomalies	Systematic and precise reflection of the entire malformation Take into account associated malformations	Simple, user-friendly, widely used
Limitations	Recently introduced; clinical utility will be determined over time.	Unwieldy for use in clinical practice with difficulties in assignment to specific classes	More complex to use clinically Doesn't suggest the aethiopathogenicity	Lack of classification for complex malformations resulting from a dependence of classification systems for uterus, cervix and vagina.

Based on [Grimbizis et al., 2013; Acién and Acién, 2011; Oppelt et al., 2005; The American Fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, müllerian anomalies and intrauterine adhesions, 1988]

2. CANDIDATE GENES INTERROGATED IN COHORTS WITH UTERINE MALFORMATIONS

Supplemental data Table 2. Candidate genes interrogated in cohorts with uterine malformations by targeted sequencing or WES

(Negative results are mentioned only for targeted sequencing)

Gene	*Reference*	*Type of study*	*Number of positive/ total number of individuals in the cohort*	*Variant of unknown significance*[*]	*Disrupting variants*[§]	*Uterine phenotypes in positive/ # with isolated UM*
AMH	[Zenteno et al., 2004]	Targeted	**0/15**	3	**0**	U5/3
	[Mikhael et al. 2021]	WES	**3/111**[1]			
AMHR2	[Zenteno et al., 2004]	Targeted	**0/15**	0	**0**	-
BMP4	[N. Chen et al. 2021]	WES	**3/592**	1	**2**	U5/2
BMP7	[N. Chen et al. 2021]	WES	**2/592**	1	**1**	U5/2
CFTR	[Timmreck et al., 2003a]	Targeted	**0/25**	4[2]	**2**	U5/7
	[Mikhael et al. 2021]	WES	**9/111**			
CTNNB1	[Drummond et al., 2008]	Targeted	**0/12**	0	**0**	0
DACT1	[Xing et al. 2016]	Targeted	**2/16**	2	**0**	U3/NR
DLGH1	[Ravel et al., 2012]	Targeted	**0/12**	-	**-**	-
EMX2	[Liu et al., 2015]	Targeted	**1/517**	4	**2**	U3bC2 or U5/2
	[Li et al. 2022]	Targeted	**4/40**			
	[N. Chen et al. 2021]	WES	**1/592**			
ESR1	[Brucker et al. 2017]	Targeted	**2/93**	2	**0**	U5/2
GALT exon 10	[Zenteno et al., 2004]	Targeted	**0/15**	1	**0**	U5/0
	[Mikhael et al. 2021]	WES	**1/111**			
GEN1	[Wang 2020]	Targeted	**5/125**	4	**1**	U5/ ?
GREB1L	[Jolly et al 2023]	WES	**15/590**	23	**10**	U5/9
	[Jacquinet et al. 2020]	WES	**4/9 families**			
		Targeted	**4 /68**			
	[Herlin 2020] ;[De Tomasi 2017] ;[Sanna-Cherchi 2017]	WES	**CR**			
HNF1B	[Alvelos et al., 2015]; [Heidet et al., 2010]; [Shihara et al., 2004]; [Iwasaki et al., 2001]; [Lindner et al., 1999] ; [Faguer et al. 2011]; [Agarwal et al. 2022]	Targeted	**CR**	0	**12**[2]	U2,U3,U4,U5/1[3]
	[Ledig et al., 2011]	Targeted	**0/56**			
	[Oram et al., 2010]	Targeted	**8/108**[1]			
	[Bernardini et al., 2009]	Targeted	**0/20**			
	[Bingham et al., 2002]	Targeted	**2/9**[1]			
	[Williams et al. 2017]	Targeted	**0/100**			
HOXA7	[Chen et al., 2014b]	Targeted	**0/192**	0	**0**	0
	[Burel et al., 2006]	Targeted	**0/6**			
HOXA9	[Chen et al., 2014b]	Targeted	**1/192**	1	**0**	U3/1
	[Burel et al., 2006]	Targeted	**0/6**			
HOXA10	[Ekici et al., 2013]	Targeted	**4/27**	7[2]	**1**	U2,U3,U5/5
	[Cheng et al., 2011]	Targeted	**1/109**			
	[Liatsikos et al., 2010]	Targeted	**0/30**			
	[Lalwani et al., 2008]	Targeted	**0/26**			
	[Burel et al., 2006]	Targeted	**0/6**			
	[Mikhael et al. 2021]	WES	**1/111**			
	[N. Chen et al. 2021]	WES	**1/592**			
HOXA11	[Chen et al., 2014a, 11]	Targeted	**4/192**	5[2]	**0**	U2/4
	[Liatsikos et al., 2010]	Targeted	**1/30**			
	[Burel et al., 2006]	Targeted	**0/6**			
HOXA13	[Imagawa et al., 2014]	Targeted	**CR**	0	**5**	U2,U3/0
	[Ekici et al., 2013]	Targeted	**0/27**			
	[Parker et al., 2011]	Targeted	**CR**			
	[Jorgensen et al., 2010]	Targeted	**CR; 0/17**			
	[Utsch et al., 2007]	Targeted	**CR**			
	[Burel et al., 2006]	Targeted	**0/6**			
	[Innis et al., 2004]	Targeted	**CR**			
	[Goodman et al., 2000]	Targeted	**CR**			
	[Mortlock and Innis, 1997]	Targeted	**CR**			
LAMC1	[Ravel et al., 2012]	Targeted	**0/12**	4	**0**	U5/3
	[Mikhael et al. 2021]	WES	**4/111**			

LHX1	[Sandbacka et al., 2013]	Targeted	**5/112**	5[2]	**2**	U5/7
	[Xia et al., 2012]	Targeted	**1/96**			
	[Ledig et al., 2012]	Targeted	**1/62**			
	[Ledig et al., 2011]	Targeted	**1/56**			
	[Bernardini et al., 2009]	Targeted	**0/20**			
	[Williams et al. 2017]	Targeted	**0/100**			
	[Zhang et al. 2017]	WES	**1/10**			
LRP10	[Mikhael et al. 2021]	WES	**4/111**[6]	4	**0**	U5/3
MMP14	[Mikhael et al. 2021]	WES	**2/111**	2	**0**	U5/2
OXTR	[Brucker et al. 2017]	Targeted	**2/93**	1	**0**	U5/0
PAX2	[Wang et al., 2012]	Targeted	**1/192**	2[4]	**0**	U3,U5/2
	[Mikhael et al. 2021]	WES	**1/111**			
PAX8	[N. Chen et al. 2021]	WES	**10/592 ;1CR**	3	**8**	U5/7
PBX1	[Ma et al., 2011]	Targeted	**0/192**	0	**0**	0
	[Burel et al., 2006]	Targeted	**0/6**			
RBM8A	[Tewes et al., 2015]	Targeted	**3/167**	2	**0**	U5/2
RARA	[Mikhael 2021]	WES	**2/111**	3	**0**	U5/2
RARG	[Cheroki et al., 2006]	Targeted	**0/25**	0	**0**	0
RXRA	[Cheroki et al., 2006]	Targeted	**0/25**	0	**0**	0
SHOX	[Mikhael 2021]	WES	**2/111**	2	**0**	U5/1
TBX6	[Tewes et al., 2015]	Targeted	**6/167**	11	**11**	U5, U2, U3/26
	[Sandbacka et al., 2013]	Targeted	**17/112**	2 (possible		
	[Tewes et al. 2019]	Targeted	**4/125**	functional		
	[N. Chen et al. 2021]	WES	**2/592**	polymorphism)		
	[C. Ma et al. 2022]	WES	**15/622**			
	[Mikhael et al. 2021]	WES	**3/111**			
TP63	[Wang et al. 2016]	Targeted	**1/200**	0	**1**	U4/1
WNT4	[Waschk et al., 2015]	Targeted	**0/14**	8	**3**	U2,U4,U5/4
	[Chang et al., 2012]	Targeted	**1/189**			
	[Philibert et al., 2011]	Targeted	**1/4**			
	[Ravel et al., 2009]	Targeted	**1/11**			
	[Philibert et al., 2008]	Targeted	**1/25**			
	[Biason-Lauber et al., 2007]	Targeted	**1/6**			
	[Cheroki et al., 2006]	Targeted	**1/25**			
	[Clément-Ziza et al., 2005]	Targeted	**0/19**			
	[Biason-Lauber et al., 2004]	Targeted	**CR**			
	[Williams et al. 2017]	Targeted	**0/100**			
	[Mikhael et al. 2021]	WES	**3/111**			
	[L. Li et al. 2021]	WES	**1/12**			
	[Ragitha et al. 2023]	Targeted	**0/103**			
WNT5A	[Wu et al., 2013]	Targeted	**1/189**	1	**0**	U4/1
	[Ravel et al., 2009]	Targeted	**0/11**			
WNT7A	[AlQattan et al., 2013]	Targeted	**CR**	2	**2**[5]	U3,U5/1
	[Dang et al., 2012]	Targeted	**1/191**			
	[Eyaid et al., 2011]	Targeted	**CR**			
	[Ravel et al., 2009]	Targeted	**1/11**			
	[Timmreck et al., 2003b]	Targeted	**0/40**			
WNT9B	[Mikhael et al. 2021]	WES	**1/111**	9	**3**	U3,U5/9
	[Waschk et al., 2015]	Targeted	**6/226**			
	[Wang et al., 2014]	Targeted	**2/42**			
	[Tang et al., 2014]	Targeted	**0/191**			
	[Ravel et al., 2009]	Targeted	0/11			
	[N. Chen et al. 2021]	WES	**1/592**[6]			
	[Wang et al. 2020]	Targeted	**2/125**			

Uterine phenotypes are reported following the ESHRE/ESGE classification (U5 = aplastic uterus, U4 = hemi-uterus, U3 = bicorporeal uterus, U3bC2 = complete bicorporeal uterus with a double cervix (formerly didelphys uterus), U2 = septate uterus); CR = case report

*Variant of unknown significance: Missense variant predicted to be damaging, splicing variant, nucleotide deletion predicted inframe, variant in 3' or 5' UTR (without functional characterization)

§Disrupting variants: truncating variants, missense variant with confirmed disrupting function by functional analysis, missense variants reported multiple times, canonical splicing variant

[1]One variant in the publication was not considered as the population frequency of this variant is too high (4%).

[2] One variant in several individuals

[3] One patient presented with isolated uterine malformation. However, her relatives presented with symptoms of RCAD.

[4] Location of the variant was unclear based on the publication [Wang 2012].

[5] Autosomal recessive syndromic cases.

[6] One of the variant is in the non canonical transcript

Supplemental data Table 3. Syndromes associated with uterine malformations

(Syndromes associated with hypoplastic uterus (i.e., 46,XX ovarian dysgenesis or hypogonadotropic hypogonadism) are not included).

SYNDROME (OMIM IDENTIFICATION)	*FEATURES*	*UTERINE ANOMALIES*	*CLINICAL CASE REPORTS (LITERATURE) (non exhaustive)*	*GENES*	*INHERITANCE*
MULTIPLE CASE REPORTS-AT LEAST ONE CASE REPORTED WITH A GENE MUTATION					
Craniofrontonasal syndrome	Frontonasal dysplasia, craniofacial asymmetry, craniosynostosis, bifid nasal tip, grooved nails, abnormalities of the thoracic skeleton, anomalies of the extremities, rarely corpus callosum agenesis, diaphragmatic hernia. Normal intelligence. Female more affected than male	Bicorporeal uterus	**Gürsoy 2021** **Bukowska-Olech 2021** **Acosta-Fernández 2020**	***EFNB1***	X-linked dominant
Currarino Syndrome (#176450)	Anorectal abnormalities, partial sacral agenesis, and presacral mass	Bicorporeal uterus, aplastic uterus, uterus duplication, septate uterus	O'Riordain et al., 1991; Lynch et al., 2000; Belloni et al., 2000; Martucciello et al., 2004; **Ciotti et al., 2011**; Ohno et al., 2013; Shin 2019	***MNX1***	AD
Donnai-Barrow syndrome (# 222448)	Variable :Facial features (hypertelorism), ocular complications (high myopia, retinal detachment, retinal dystrophy), agenesis of the corpus callosum, sensorineural hearing loss, intellectual disability, and congenital diaphragmatic hernia and/or omphalocele, proteinuria	Bicorporeal uterus	Pober 2009 **Yuan et al, 2023**	***LRP2***	AR
Fraser Syndrome (#219000)	Cryptophtalmos, nose and ear anomalies, laryngeal stenosis, renal agenesis/hypoplasia, intellectual disability, ambiguous external genitalia	Bicorporeal uterus, hemi-uterus, aplastic uterus	Slavotinek and Tifft, 2002; **Slavotinek et al., 2006**; **Shafeghati et al., 2008**; Kalaniti and Sandhya, 2011; Tessier 2016	***FRAS1,FREM1,*** *GRIP1*	AR
Hand-foot-genital syndrome (#140000)	Small feet with short great toes and abnormal thumbs, genital anomalies, ectopic accessory ureteral orifice, vesicoureteral reflux, ureteropelvic junction obstruction	Various degrees of bicorporeal uterus with or without two cervices or a longitudinal vaginal septum	**Goodman et al., 2000; Mortlock and Innis, 1997; Innis et al., 2004; Utsch et al., 2007; Jorgensen et al., 2010; Parker et al., 2011; Imagawa et al., 2014**	***HOXA13***	AD
HDR (Hypoparathyroidism, Sensorineural Deafness and Renal anomaly) syndrome (#146255)	Symptomatic or asymptomatic hypocalcemia, undetectable or low level of serum parathyroid hormone (PTH), bilateral sensorineural deafness, kidney anomalies (cysts, hypoplasia/aplasia), vesicoureteral reflux, glomerular nephropathy and nephrotic syndrome	Bicorporeal uterus, aplastic uterus, septate uterus	**Nakamura et al., 2011; Moldovan et al., 2011; Hernández et al., 2007; Vallejo-Urrego 2019**	***GATA3***	AD

Müllerian aplasia and hyperandrogenism (#158330)	Hyperandrogenism, pelvic kidney, renal agenesis	Aplastic uterus	**Philibert et al., 2011, 2008; Biason-Lauber et al., 2007, 2004**	***WNT4***	AD
MYRF-Related Cardiac Urogenital syndrome (#618280)	Variable:Hyperopia, nanophtalmos, congenital heart defect,congenital diaphragmatic hernia, pulmonary hypoplasia, ambiguous genitalia/persistent müllerian ducts remnants in males, Typical cognition and development to severely delayed, no recognizable dysmrophic features. Absent or small ovaries, horseshoe kidney and hydronephrosis (reported in 1 individual)	Aplastic uterus and fallopian tubes with blind-ending vagina	**Qi 2018** **Hamanaka 2019**	***MYRF***	AD
Renal Cysts and Diabetes Syndrome (#137920)	Abnormal liver function, early-onset diabete mellitus, hypomagnesemia, pancreatic hypoplasia, hyperuricemia, congenital anomalies of the kidney and urinary tract	Aplastic uterus, bicorporeal uterus	**Bingham et al., 2002; Shihara et al., 2004; Harries et al., 2005; Edghill et al., 2008; Oram et al., 2010; Iwasaki et al., 2001; Lindner et al., 1999; Carbone et al., 2002; Edghill et al., 2006**	***HNF1B***	AD
Smith-Lemli-Opitz (#270400)	Prenatal and postnatal growth retardation, microcephaly, intellectual disability, distinctive facial features, cleft palate, cardiac defects, underdeveloped external genitalia in males, postaxial polydactyly, 2-3 syndactyly of the toes	Bicorporeal uterus	**Quélin et al., 2012;** Lowry et al., 1968	***DHCR7***	AR
STAR syndrome (Toe syndactyly, Telecanthus, anogenital and renal malformations syndrome) (#300707)	Telecanthus, clinodactyly 5th finger, syndactyly of toes, anal atresia, genital anomalies (hypolastic labia, duplicated vagina), VU reflux, kidney agenesis, small height.	Bicorporeal uterus, duplicated uterus	**Unger et al., 2008**	***CCNQ***	X-linked
Syndromic ciliopathies (#616258, #236700, #236680,#209900)	**Bardet-Biedel:** intellectual disability, pigmentary retinopathy, polydactyly, obesity, hypogonadotropic hypogonadism **McKusick-Kaufman:** polydactyly, genitourinary malformation (hydrometrocolpos-transverse vaginal septum), heart and gastro-intestinal malformations **Hydrocephalus**: hydrocephaly, micrognathia, cleft lip/palate, malformed respiratory tract, congenital heart defects, club feet, polydactyly	Aplastic uterus, bicorporeal uterus, uterine hypoplasia	**Filges et al., 2014** (fetal ciliopathy-MKS12) Witters et al., 2012 (MKKS) **Thomas et al., 2012** (fetal ciliopathy with feature of MKS and OFDIV) **Deveault et al., 2011** (BBS- (vaginal agenesis)) Salonen and Herva, 1990 (Hydrolethalus) Stoler et al., 1995; Campo and Aaberg, 1982 (BBS)	***KIF14,TCTN3, BBS12*, NEK8***	AR

	Meckel syndrome: encephalocele, postaxial polydactyly, dysplastic polycystic kidneys, male pseudohermaphrodism **Meckel syndrome 12:** intrauterine growth retardation, severe microcephaly, renal cystic dysplasia/agenesis, and complex brain and genitourinary malformations **Mohr-Majewski syndrome (OFD IV):** bone dysplasia, tibial defect, renal anomalies, brain anomalies **Renal-Hepatic-Pancreatic Dysplasia 2:**heart malformation,situs inversus,hepatic fibrosis,cystic dysplasia of the liver/pancreas,renal cystic dysplasia/enlarged kidneys		**Frank 2013 ; Grampa 2016 (Renal-Hepatic-Pancreatic Dysplasia 2**		
Syndromic microphtalmia type 12 and type 9 (Matthew-wood syndrome; PDAC syndrome) (#615524; #601186)	Severe pulmonary aplasia or dysgenesis or hypoplasia, diaphragmatic eventration or hernia, anophthalmia or microphthalmia or ocular dysplasia, cardiac defect.	Bicorporeal uterus, hypoplastic uterus	Chitayat et al., 2007 ; **Srour et al., 2013**	*STRA6,* ***RARB***	AR, AD
SPECC1L hypertelorism syndrome (Opitz G/BBB syndrome type II; Teebi hypertelorism syndrome) (#145410; %145420)	Hypertelorism, umbilical hernia, craniosynostosis, cleft lip and palate, prominent forehead, broad nasal root, aortic root dilation	Bicorporeal uterus	**Bhoj et al., 2015; Kruszka et al., 2015**	***SPECC1L***	AD
Townes-Brocks (#107480)	Imperforate anus, dysplastic ears (overfolded superior helices and preauricular tags; frequently associated with sensorineural and/or conductive hearing impairment), thumb malformations, renal anomalies	Bicorporeal uterus, septate uterus	**Botzenhart et al., 2005;** Johnson et al., 1996, Webb 2017	***SALL1*** ***DACT1***	AD
Ulnar and fibula syndrome, with severe limb deficiency (#276820)	Absence or reduction of limbs, facial abnormalities, pelvic and genital abnormalities, horseshoe kidneys, unilateral renal agenesis, hydronephrosis	Aplastic uterus	**Eyaid et al., 2011; AlQattan et al., 2013;** Farag et al., 1993; Teebi, 1993	***WNT7A***	AR
Wiedemann-Steiner (#605130)	Mild to moderate ID, short stature, failure to thrive,dysmorphism, hypertrichosis, vertebral anomalies, renal/heart malformation	Aplsatic uterus	**Sheppard et al., 2021**	***KMT2A***	AD
Woodhouse-Sakati syndrome (#241080)	Alopecia, diabetes mellitus, hypogonadism, deafness, cognitive decline, extrapyramidal features	Aplastic uterus, uterine hypoplasia	**Steindl et al., 2010**	***DCAF17***	AR
SINGLE CASE REPORT WITH AN IDENTIFIED GENE					
Cenani-Lenz syndrome (prenatal lethal form) (#212780)	Syndactyly and oligodactyly, partial to complete fusion of the carpals, metacarpals, or phalanges, radioulnar synostosis,	Aplastic uterus	**Lindy et al., 2014**	***LRP4,****APC*	AR

	mesomelic shortening, prominent forehead, hypertelorism, downslanting palpebral fissures, and micrognathia, renal agenesis/hypoplasia				
Chondrodysplasia, acromelic, with genital anomalies (#609441)	Short stature, acromesomelic shortness of the limbs, hand/foot malformations, absence of ovaries	Aplastic uterus	**Demirhan et al., 2005**	***BMPR1B***	AR
Cousin syndrome (#260660)	Short stature, short limb, macrocephaly (real or relative), dysmorphic features, humeroradial synostosis, hips dislocation, scapular and iliac hypoplasia	Arcuate uterus	**Dikoglu et al., 2013**	***TBX15***	AR
Lymphoedema-distichiasis syndrome (#153400)	Distichiasis, primary lymphoedema of pubertal onset	Double uterus	**Brice et al., 2002**	***FOXC2***	AD
Limb mammary syndrome (#603543)	Ectrodactyly, mammary-gland and nipple hypoplasia, cleft palate without cleft lip	Aplastic uterus	**Guazzarotti et al., 2008**	***TP63***	AD
Leukodystrophy, hypomyelinating, 2 (#608804)	Nystagmus, impaired motor development, ataxia, choreoathetotic movements, dysarthria, and progressive spasticity, renal agenesis	Aplastic uterus	**Yalcinkaya et al., 2012**	***GJC2***	AR
Serpentine fibula polycystic kidneys (Hajdu-Cheney syndrome) (#102500)	Craniofacial abnormalities (prominent eyes, full cheeks, arched eyebrows, and micrognathia), skeletal abnormalities (wormian bones, fibular bowing, and osteoporosis), polycystic kidneys	Bicorporeal uterus	**Martin et al., 2014**	***NOTCH2***	AD
SOPH (short stature, optic nerve atrophy, and Pelger-Huet anomaly) syndrome (#614800)	Postnatal growth failure, loss of visual acuity, Pelger-Huët anomaly, craniofacial features (Yakuts population)	Hypoplastic uterus	**Maksimova et al., 2010**	***NBAS***	AR
Tetra-amelia syndrome, autosomal recessive (#273395)	Tetra-amelia, cleft lip/palate, gastroschisis, diaphragmatic defect, lung anomaly, adrenal gland agenesis, spleen agenesis, ophtalmological anomalies, malformed nose, choanal atresia, atresia of urethra, vagina and anus, hypoplasia of pelvis, abnormal externe genitalia, renal agenesis	Malformed uterus	**Niemann et al., 2004**	***WNT3***	AR
Ulnar-mammary syndrome (#181450)	Posterior limb deficiencies or duplications, apocrine/mammary gland hypoplasia and/or dysfunction, abnormal dentition, delayed puberty in males, genital anomalies	Bicorporeal uterus	**Sasaki et al., 2002**	***TBX3***	AD

≥2 CASE REPORTS NOT INVESTIGATED MOLECULARLY (BUT ETIOLOGY KNOWN FOR THE SYNDROME)					
Apert syndrome (#101200)	Craniosynostosis, midface hypoplasia, finger and toe anomalies and/or syndactyly	Bicorporeal uterus	Blank, 1960; Lindsay et al., 1975	*FGFR2*	AD
Basal cell nevus syndrome (Gorlin syndrome) (#109400)	Multiple early-onset basal cell carcinoma (BCC), odontogenic keratocysts, skeletal abnormalities	Bicorporeal uterus, septate uterus, infantilis uterus/uterine hypoplasia	Kimonis et al., 1997; Ramaglia et al., 2006; Kohli et al., 2010	*PTCH1, PTCH2, SUFU*	AD
Beckwith-Wiedemann syndrome (#130650)	Macrosomia, macroglossia, visceromegaly, embryonal tumors, omphalocele, neonatal hypoglycemia, ear creases/pits, adrenocortical cytomegaly, and renal abnormalities.	Bicorporeal uterus	Weng et al., 1995; Irving, 1967	*Loss of methylation IC2 (maternal allele); paternal UPD of 11p15; gain of methylation IC1(maternal allele); CDKN1C mutations*	AD
Coffin-Siris syndrome (#135900)	Aplasia or hypoplasia of the distal phalanx or nail of the fifth digit, distinctive facial features, moderate to severe developmental/cognitive delay	Aplastic uterus	Goyal et al., 2010; Coffin and Siris, 1985	*ARID1A, ARID1B, SMARCA4, SMARCB1, or SMARCE1*	AD
Goltz syndrome (#305600)	Focal dermal hypoplasia, asymetric limbs anomalies, dental, nails and hair anomalies, asymetry of face, dysmorphic features, eyes anomalies, kidney anomalies	Bicorporeal uterus	Lopez-Porras et al., 2011; Reddy and Laufer, 2009	*PORCN*	X-linked
Linear skin defects with multiple congenital anomalies 1 (MIDAS syndrome) (#309801)	Unilateral or bilateral microphthalmia, linear skin defects (aplastic skin, hyperpigmented area) limited to the face and neck	Bicorporeal uterus, rudimentary uterus	Happle et al., 1993; Anguiano et al., 2003	*HCCS*	X-linked
Neu-Laxova syndrome (#256520)	Severe fetal growth restriction, microcephaly, distinct facial appearance, ichthyosis, skeletal anomalies, perinatal lethality	Bicorporeal uterus, hemi-uterus	Coto-Puckett et al., 2010; Ostrovskaya and Lazjuk, 1988	*PHGDH*	AR
Peters-Plus syndrome (#261540)	Anterior chamber eye anomalies, short limbs with broad distal extremities, variable developmental delay/intellectual disability, exaggerated Cupid's bow of the upper lip, short palpebral fissures, ear anomalies, cleft lip/palate.	Rudimentary uterus	Thompson et al., 1993; Streeten et al., 1983; Maillette de Buy Wenniger-Prick and Hennekam, 2002	*B3GALTL*	AR
Popliteal pterygium syndrome (#119500)	Cleft lip, a cleft palate, fistulae of the lower lip, Webbing of the skin extending from the ischial tuberosities to the heels, bifid scrotum and cryptorchidism, hypoplasia of the labia majora, syndactyly of fingers and/or toes, anomalies of the skin around the nails	Bicorporeal uterus, hypoplastic uterus	Bartsocas and Papas, 1972 ; Gorlin et al., 1968	*IRF6*	AD
TAR syndrome (#274000)	Bilateral absence of the radii with presence of both thumbs, transient thrombocytopenia, kidney anomalies	Aplastic uterus	Griesinger et al., 2005; Behera et al., 2005; Childs and Hall, 2005; Ahmad and Pope, 2008; Rall et al., 2015	Del1q21+ *RBM8A*	AR

CHROMOSOMAL SYNDROMES (≥ 2 CASE REPORTS)					
22q11.2 deletion (#188400)	Growth retardation, intellectual disability or learning difficulties	Aplastic uterus	Cheroki et al., 2006; Devriendt et al., 1997; Sundaram et al., 2007; Uliana et al., 2008; Morcel et al., 2011	Chromosomal	AD
17q12 deletion (#614527)	Developmental delay, learning disability, autism or autistic features, kidney and urinary tract problems, diabetes, behaviour concerns, facial features	Aplastic uterus, bicorporeal uterus	Cheroki et al., 2008; Nik-Zainal et al., 2011; Bernardini et al., 2009; Moreno-De-Luca et al., 2010	Chromosomal	AD
Cat-eye syndrome (#115470)	Anal atresia, iris coloboma, downward-slanting palpebral fissures, preauricular pits/tags, cardiac defects, kidney problems, short stature, scoliosis/skeletal problems, intellectual disability, micrognathia, cleft palate	Aplastic uterus	Cullen et al., 1993; Schinzel et al., 1981	Chromosomal (trisomy or tetrasomy 22pter-22q11)	AD
Pallister-Killian syndrome (#601803)	Profound intellectual disability, seizures, streaks of hypo- or hyper-pigmentation, facial anomalies (prominent forehead with sparse anterior scalp hair, flat occiput, hypertelorism, short nose with anteverted nostrils, flat nasal bridge, short neck)	Aplastic uterus, hypoplastic bicorporeal uterus	Reynolds et al., 1987; Bernert et al., 1992	Chromosomal (Tetrasomy 12p mosaic)	Somatic mosaicism
Turner Syndrome	Short stature, gonadal dysgenesis, cardiovascular malformations, oedema of the hands and feet, renal anomalies	Rudimentary uterus, infantilis uterus/uterine hypoplasia, aplastic uterus	Wonkam et al., 2015;Vaddadi et al., 2013; Bakalov et al., 2007	Chromosomal (45,X)	Sporadic
Trisomy 13 (Patau syndrome) *Microcephaly, microphthalmia, malformed ears, cleft lip and palate, cardiac anomalies, polydactyly, intrauterine growth retardation, severe developmental retardation*	Holoprosencephaly, microphtalmia/anophtalmia, cutis aplasia, cleft lip/palate, cardiac malformation, postaxial polydactyly, omphalocele, kidney malformations, severe/profound intellectual disability, growth retardation.	Bicorporeal uterus, hemi-uterus, septate uterus, arcuate uterus	Moerman et al., 1988; Baty et al., 1994	Chromosomal	Sporadic
Trisomy 18 (Edwards' syndrome)	Intrauterine growth retardation, congenital heart defect, renal defects, short sternum, prominent occiput, micrognathia, malformed ears, overlapping fingers, severe to profound developmental delay	Bicorporeal uterus	Baty et al., 1994; Kinoshita et al., 1989	Chromosomal	Sporadic
Wolf-Hirschhorn syndrome (#194190)	Pre- and postnatal growth deficiency, developmental disability, characteristic craniofacial features ('Greek warrior helmet' appearance of the nose), seizure disorder	Aplastic, hypoplastic uterus	Johnson et al., 1976	Chromosomal (deletion 4p16.3)	Sporadic

SYNDROMES AND ASSOCIATIONS OF UNKNOWN/HETEROGENEOUS ETIOLOGY (≥3 CASES REPORTED)					
Acro-renal-mandibular syndrome (200980)	Ectrodactyly, limb deficiency, mandibular hypoplasia, bilateral renal agenesis, cystic dysplasia	Bicorporeal uterus, hemi-uterus	Sanganalmatha et al., 2014; Evans et al., 2000; Halal et al., 1980	/	/
Caudal duplication syndrome (#607864)	Varied extent of duplication of the colon, rectum, anus, urinary system, lower genital tract, and external genitalia; spinal anomalies; kidney anomalies; abdominal wall defects	Duplication of the female genital tract	Acer et al., 2013; Shah and Joshi, 2006; Dominguez et al., 1993	*AXIN1 (hypermethylation of the promotor in 1 case)*	/
Caudal dysgenesis spectrum (including sirenomelia) (#600145)	Variable association of anomalies of the caudal spine and spinal cord, the hindgut, the urogenital system, and the lower limbs.	Bicorporeal uterus, aplastic uterus	Tica et al., 2013 (Sirenomelia[1]), Harewood et al., 2010 (Caudal dysgenesis), Bashyal et al., 2012 (Pelvis dysplasia-urogenital abnormalities) Lecoquierre 2020, **Stevens 2021**	***CDX2***	/
Fryns syndrome (229850)	Coarse face, abnormal ear shape, CL/CP, large mouth, microretrognathia, broad nasal bridge, anteverted nares, diaphragmatic defects, distal digital hypoplasia, genital tract anomalies, malformation CNS, abnormal lung lobations, cystic dysplasia of kidneys	Bicorporeal uterus	Slavotinek, 2004	/	/
Hypergonadotrophic hypogonadism and partial alopecia (241090)	Hypergonadotrophic hypogonadism, partial alopecia, abnormal gonads (streak ovaries, absence)	Aplastic/ hypoplastic uterus	Al-Awadi et al., 1985; Mégarbané et al., 2003; Tatar et al., 2009	/	/
MURCS association (601076)	cervicothoracic somite dysplasia, renal aplasia, ectopic kidney	Aplastic uterus, bicorporeal uterus	Braun-Quentin et al., 1996	/	/
Oculo-auriculo-vertebral complex (Hemifacial microsomia, Goldenhar syndrome) (164210)	Hypoplastic malar, maxillary and mandibular regions, microtia, hemivertebrae, hypoplastic vertebrae, kidney and urinary tract anomalies	Aplastic uterus	Wulfsberg and Grigbsy, 1990; Pillay et al., 2003	/	/
OEIS complex (258040)	Omphalocele, exstrophy of the cloaca, imperforate anus, and spinal defects, ambiguous genitalis, kidney anomalies	Bicorporeal uterus	Dutta, 2014; Sawaya et al., 2010	/	/
Pseudotrisomy 13 syndrome (264480)	Holoprosencephaly, severe facial anomalies, postaxial polydactyly, heart defect	Bicorporeal uterus, duplicated uterus, hypoplastic uterus	Ramos-Arroyo et al., 1994	/	
Schinzel-phocomelia (276820)	Skull defect, phocomelia, vesicoureteral reflux	Aplastic uterus	Schinzel, 1990; Froster et al., 1996; Chitayat et al., 1993	/	/

Spondylocostal dysostosis (Casamassima-Morton-Nance syndrome) (271520)	Spondylocostal dysostosis, anal atresia and urogenital anomalies (absence of vagina and external genitalia)	Bicorporeal uterus, aplastic uterus	Poor et al., 1983; Daïkha-Dahmane et al., 1998; Thauvin-Robinet et al., 2007; Sasaki et al., 2011	/	/
VATER/VACTERL Association (#192350)	Vertebral defects, anal atresia, tracheoesophageal fistula with esophageal atresia, radial dysplasia, cardiac malformations, limb anomalies, kidney anomalies	Aplastic uterus, hemi-uterus	Linke et al., 2005; Orstavik et al., 1992; Komura et al., 2007; Nunes et al., 2009; Jessel and Laufer, 2013	*ZIC3* *WBP11*	/

Review of the literature based on OMIM database, the Winter-Baraitser Dysmorphology Database and Smith's Recognizable Patterns of Human Malformation: Expert Consult - Online and Print, 7e, 2013
References in bold when some mutations were reported; genes in bold when mutations have been reported in some individuals with uterine malformations.
* 69 genes have been indexed in OMIM as associated with human ciliopathies.
[1] antiepileptic drugs during the pregnancy.

RATIONALE AND AIM OF THE THESIS

RATIONALE AND AIM OF THE THESIS

Uterine malformations, and associated renal malformations, can have a major impact on the health and social wellbeing of affected patients. Little research has been devoted to the understanding of the etiologies of uterine malformations compared to other organs and specific causes remain largely unknown so far. It is only by understanding these causes that we will understand who exactly is at risk, and will possibly be able to identify factors that may be modifiable for those at risk (eg., folate supplementation for neural tube defects). The unexplained occurrence of these malformations in multiple family members suggests that strong genetic contributors remain to be defined. In such families, kidney malformations are often present as well, supporting shared developmental pathways or interdependent development of the two organs. Thus, genes already implicated in congenital anomalies of the kidney and urinary tract (CAKUT) may also play a role in uterine development and be causal in some of these families. Studies in CAKUT have demonstrated considerable genetic heterogeneity, with about 60 genes identified so far explaining 11-20% of cases (198,323). Similarly, uterine malformations may be highly heterogeneous.

The aim of this PhD project is to identify new genes and pathways involved in uterine development. Given the frequent co-occurrence of kidney and uterine malformations, we anticipate that causal variants might be present in genes previously associated with congenital anomalies of the kidney and urinary tract (CAKUT). Whole-exome sequencing is the most cost-effective approach to examine both known CAKUT genes and to potentially identify new candidate genes in a hypothesis-free manner. Moreover, uterine malformations have rarely been explored using WES compared to other organs, reinforcing the rationale for testing this approach. We hypothesize that focusing WES analyses on families with recurrent uterine and kidney malformations will allow us to identify significant genetic factors involved in uterine malformations by comparing sequencing data from affected family members.

Additional evidence will then be essential to confirm the role of any novel gene. A secondary aim, therefore, is to investigate the role of selected candidate genes in the development of the urogenital system using the zebrafish model.

RESULTS

RESULTS

I. *GREB1L* VARIANTS IN FAMILIAL AND SPORADIC HEREDITARY UROGENITAL ADYSPLASIA AND MAYER-ROKITANSKY-KÜSTER-HAUSER SYNDROME

1. SUMMARY

Nine families with recurrence of renal and uterine malformations were recruited through collaborations with Dr. A. Lehman in Vancouver, Canada, Dr. D. Guerrier from INSERM Rennes, France, and Dr. Boucher in Besançon, France. Additionally, Dr. Guerrier provided access to DNA samples from 79 individuals diagnosed with congenital uterine anomalies, MRKH syndrome or uterovaginal aplasia. To identify potential copy number variations, chromosomal microarray analysis (180k) was performed on DNA from one affected member per family. Whole exome sequencing was then conducted on two to three individuals per family, focusing on affected members due to the frequent observation of incomplete penetrance in CAKUT and hereditary urogenital adysplasia. The goal was to identify shared genetic factors responsible for the malformations among affected relatives. Variant annotation and filtering were performed using several software tools, including Alissa Interpret (Agilent Technology). Variants were prioritized based on criteria such as allele frequency in the general population (MAF < 0.5%), inheritance pattern (zygosity), and computational predictive data (Figure 21). Initially, variants were sought in a predefined list of genes known to be associated with renal or uterine malformations in humans or animal models (Figure 21). The analysis was then expanded to include genes not previously linked to these anomalies. The ACMG/AMP guidelines were used to assess the pathogenicity of variants in known human CAKUT/CUA genes.

In this first manuscript, published in Clinical Genetics in 2020, we identified the gene *GREB1L* as a significant contributor to MRKH syndrome/uterovaginal aplasia. Likely pathogenic or pathogenic heterozygous variants in *GREB1L* were found in three of the nine families studied, with an additional family exhibiting a variant of unknown significance. Sequencing in a cohort of 68 individuals with congenital uterine malformations identified five further variants of unknown significance, with two predicted to be deleterious *in silico* based on CADD scores >25 and the alpha-missense prediction score. This publication describes the phenotypes associated with *GREB1L* variants, highlighting that uterine formation defects were the most frequent type of uterine

malformations, with renal agenesis and other CAKUT being the most common anomaly associated with *GREB1L* so far. Other associated features included skeletal anomalies and hearing loss, features that are also frequently reported in patients with MRKH syndrome. The absence of phenotype in several carriers confirmed the incomplete penetrance in some individuals.

Our publication demonstrated a potentially high diagnostic yield in families with recurrent cases of uterine and renal agenesis, as well as a possible lower, but still noteworthy, yield in sporadic cases of MRKH syndrome. Based on these findings, we would recommend sequencing *GREB1L* in families with recurrent congenital uterine malformations, particularly those involving formation defects and associating CAKUT. Additionally, testing for *GREB1L* may also be valuable in sporadic MRKH patients, especially when evaluating recurrence risk.

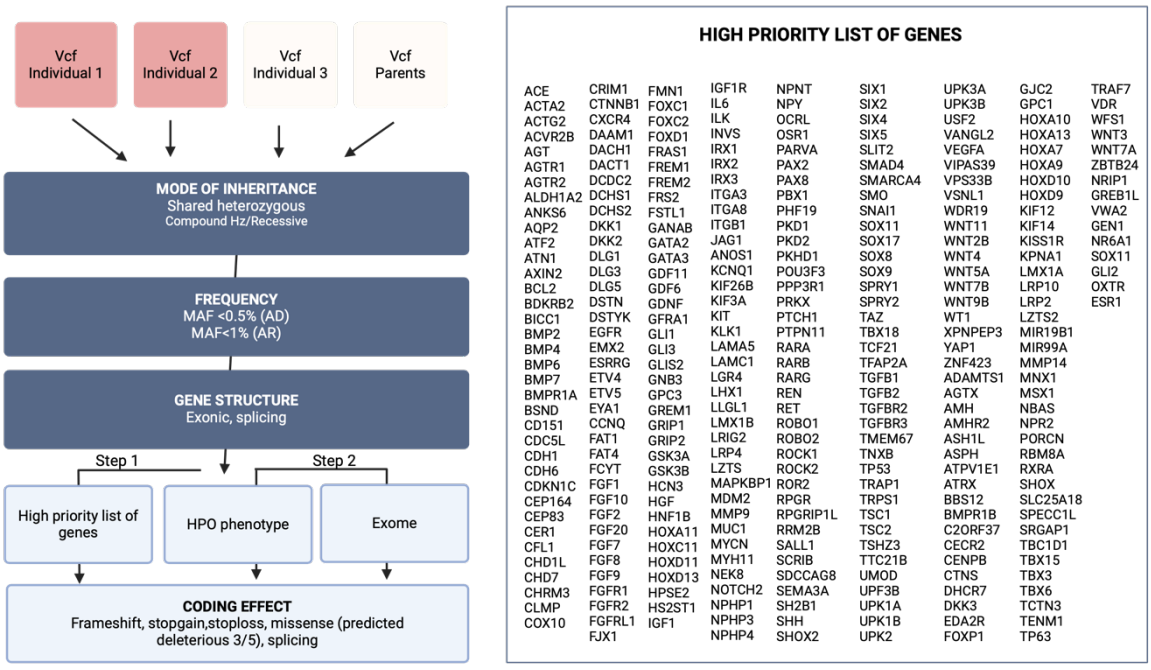


Figure 21. Workflow for the analysis of the exome data and high priority list of genes.

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ORIGINAL ARTICLE

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GREB1L variants in familial and sporadic hereditary urogenital adysplasia and Mayer-Rokitansky-Kuster-Hauser syndrome

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Abstract

Congenital uterine anomalies (CUA) may have major impacts on the health and social well-being of affected individuals. Their expressivity is variable, with the most severe end of the spectrum being the absence of any fully or unilaterally developed uterus (aplastic uterus), which is a major feature in Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH). So far, etiologies of CUA remain largely unknown. As reports of familial occurrences argue for strong genetic contributors in some cases, we performed whole exome sequencing in nine multiplex families with recurrence of uterine and kidney malformations, a condition called hereditary urogenital adysplasia. Heterozygous likely causative variants in the gene *GREB1L* were identified in four of these families, confirming *GREB1L* as an important gene for proper uterine and kidney development. The apparent mode of inheritance was autosomal dominant with incomplete penetrance. The four families included fetuses with uterovaginal aplasia and bilateral renal agenesis, highlighting the importance to investigate *GREB1L* in such phenotypes. Subsequent sequencing of the gene in a cohort of 68 individuals with MRKH syndrome or uterine malformation (mostly sporadic cases) identified six additional variants of unknown significance. We therefore conclude that heterozygous *GREB1L* variants contribute to MRKH syndrome and this probably requires additional genetic or environmental factors for full penetrance.

KEYWORDS

GREB1L, hereditary renal agenesis, Mayer-Rokitansky-Kuster-Hauser syndrome, Mullerian aplasia, renal adysplasia, renal and Mullerian duct hypoplasia, urogenital abnormalities, uterine anomalies

1 | INTRODUCTION

Congenital uterine anomalies (CUA) affect 3% of women with variable expression and severity.[1] Formation, fusion or septal absorption defects during the embryonic development result in aplastic uterus/hemi-uterus, bicorporeal uterus and septate uterus, respectively.[2] The most severe form of CUA is the congenital absence of the uterus, cervix, and the upper part of the vagina in an otherwise phenotypically normal 46, XX female, which characterizes the Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH). In its typical, complete and isolated form, this anomaly is referred to as MRKH type 1. Atypical forms

Daniel Guerrier and Vincent Bours share equal responsibility for the scientific supervision of the research.

(incomplete or symptomatic because of functional endometrial tissues) and/or the presence of additional malformations (most frequently renal or vertebral) define MRKH type 2.[3] MRKH and CUA are mainly sporadic, suggesting multifactorial/polygenic inheritance. Recently, models of semicloning technology in mice have provided first evidence for digenic/oligogenic inheritance.[4] In familial cases, strong genetic contributors might create high susceptibility with the requirement of additional genetic, epigenetic or environmental factors to explain the incomplete penetrance. So for, a few underlying genetic factors have been identified in the overall CUA. Recurrent copy number variants in 17q12, 22q11.2, 16p11.2 and 1q21.1 were implicated in both Müllerian fusion anomalies and MRKH with 7% to 14% of girls with CUA or MRKH shown to be carriers for deletion/duplication in one of these susceptibility loci.[5,6]Variants of unknown significance were reported in *LHX1*, *WNT9B* and *TBX6* genes in significant but still low number of individuals[3] and association with functional polymorphisms in *TBX6* was suggested in previous studies.[7,8] CUA have also been reported in a high number of syndromes[3] suggesting that multiple developmental genes are involved in uterine development. Prime examples of syndromes exhibiting CUA as a major feature include Mullerian aplasia and hyperandrogenism (OMIM #158330) due to *WNT4* heterozygous pathologic variants and the renal cyst and diabetes (RCAD) syndrome (OMIM: #137920) caused by *HNF1B* heterozygous mutation.

Focusing on familial cases might help identifying new genes involved in uterine development. Whereas recurrence of isolated uterine malformation is rare, several families have been reported with developmental defects of the kidney and/or urinary tracts and anomalies of the Mullerian ducts,[9] a condition referred to as hereditary urogenital adysplasia by Schimke and King in 1980.[10] The close temporal and spatial proximity of Mullerian and Wolfian ducts during development in early embryonic life suggested a common pathology basis for both defects. As it is the case for familial congenital anomalies of kidney and urinary tract (CAKUT),[11] high genetic heterogeneity is likely in CUA as well and some genes have been shown to be involved in both uterine and renal malformations in humans. First reported in families with CAKUT, *GREB1L* heterozygous variants were also identified in individuals with CUA and MRKH syndrome in some affected families.[12-14] The function of this gene and its role during kidney and female genital tract development is not yet understood. *GREB1L* (for growth regulation by estrogen in breast cancer 1 like) is part of a chromatin complex with retinoic acid and steroid hormone receptors, and its role as a coactivator in retinoic acid-mediated transcription has been suggested.[15] Animal models have confirmed its causality with absence of kidneys and genital tracts demonstrated in E13.5 knockout mice embryos.[12]

In order to identify new genes involved in CUA and MRKH, we performed whole exome sequencing (WES) in nine multiplex families with recurrence of uterine and kidney malformations. Herein, we describe four additional families with *GREB1L* variants. Subsequently, we sequenced the gene in a separate cohort of 68 individuals with MRKH or CUA. We confirm *GREB1L* as being a frequent genetic cause for hereditary urogenital adysplasia and a significant contributor in MRKH syndrome, more precisely in MRKH type 2 subgroup.

2 | MATERIALS AND METHODS

2.1 | Subjects

DNA from 39 individuals from nine families was collected after written informed consent through three different research study protocols: the genetic alterations in rare diseases (GARD) protocol, in the Department of Medical Genetics, Children's and Women's Health Center of British Columbia, Vancouver, Canada; the French national Programme de researches sur les aplasies Müllériennes (PRAM) network; the genetic alterations in CUA (GACUA) protocol, CHU of Liège, Belgium. Affected relatives presented CUA and/or kidney malformations. When possible, DNA from unaffected relatives was also collected. Clinical data for each affected relative are described in Table S1, Supporting information.

DNA extracted from saliva samples, tissue samples (from autopsy) or blood samples was available for at least two affected relatives per family. Normal chromosomal microarray performed at least in one affected family member (Agilent SNP array 180 k [Amadid 029830]) was a prerequisite.

Pedigrees are described in Figure 1 (families 5,6,7 and 9) and in Figure S1 (families 1-4 and 8).

DNA from 63 additional individuals with MRKH syndrome or uterovaginal aplasia and five with a hemi-uterus or bicorporeal uterus was collected through the PRAM network and the GACUA protocol (Table S2). The cohort included three fetal samples. DNA was extracted from blood samples or, in case of fetuses, from tissue samples. Seven of 68 individuals had a family history of one relative with kidney or uterine malformation (DNA was not available for the relatives). Clinical data for the cohort are summarized in Table 1.

2.2 | Whole exome sequencing

After library preparation using the Ion AmpliSeq Exome Kit (family 1 and 2), the Agilent SureSelect V4 kit (family 3 and 4) and the Agilent SureSelect Human All Exon V6 capture technology (family 5-9), WES was performed on Ion Proton System (family 1 and 2), Nextseq500 (family 7) or HiSeq 2000/2500 Illumina sequencers (family 3-6, 8 and 9), generating 150 bp reads (paired-end with Illumina platforms) that were aligned to the hg19 reference human genome. The average exome coverage was above ×50. Variants were annotated and filtered using Alissa Interpret v2.1 (Agilent Technologies). Based on the hypothesis of an autosomal dominant inheritance with incomplete penetrance, at first, only affected relatives were sequenced (two or three for each family). Shared heterozygous variants were filtered based on a minor allele frequency <0.1% in the population database

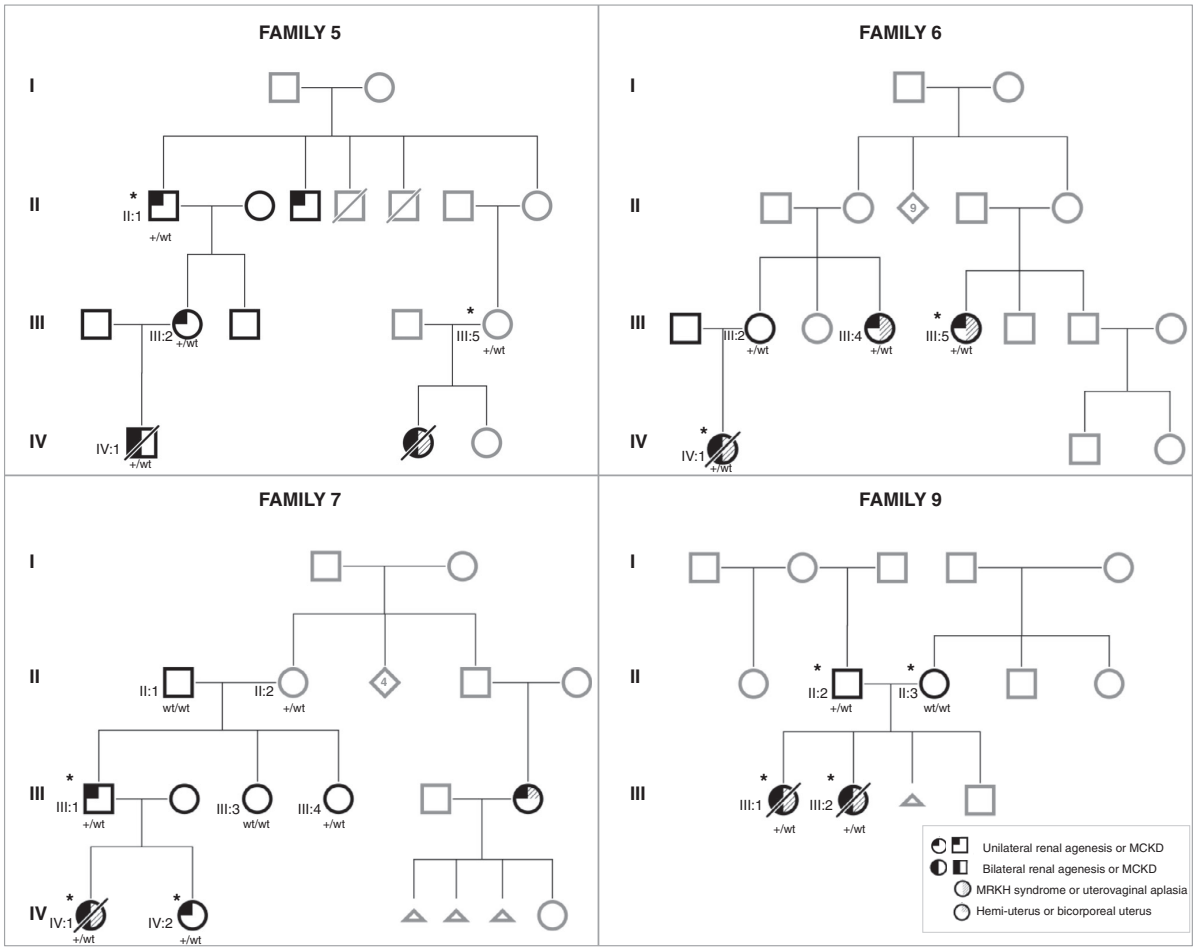

FIGURE 1 Pedigree of the four families with heterozygous *GREB1L* variants. Male and female symbols are black when the phenotype was known for the individual (renal US performed, no known uterine malformation). Male and female symbols are gray when the individual's phenotype was unknown. Symbol numbers indicates individuals with available DNA. An asterisk indicates the relatives in whom whole exome sequencing was performed. Sanger sequencing results are indicated for the individuals who were tested as follow. +/wt: heterozygous variant present; wt/wt: wild type. Pedigrees were generated using PedigreeXP (http://www.pedigreexp.com)

Gnomad,[16] their presence in exonic regions ±20 bp of exon boundaries and a predicted change at the protein level (synonymous variants excluded). The functional impact of single nucleotide variants was evaluated using several in silico prediction tools (PolyPhen-2, SIFT, PROVEAN, Mutation Taster, Mutation Assessor for missense variants; Human Splicing Finder and Alamut Visual splicing predictions tools to assess splicing effect) (Table S3). For each variant of interest, we investigated the CADD-scaled score, an integrative score based on the output of multiple in silico programs and genomic annotations that rank the variant deleteriousness relative to all single nucleotide variants in the genome (a score between 20 and 30 meaning that the variant is estimated to be in the 1% of the most deleterious variants in the genome).[17] We considered a cutoff of 25 for the CADD-scaled score as an indicative of a possible deleterious effect.[18]The GERP++RS score, estimating the evolutionary constraint for individual alignment site, was used to assess the level of conservation of modified nucleotides. A positive score ≥2 (with a maximum of 6.18) indicates evolutionary constraint and conservation.[19] Conservation of the aminoacid was evaluated through the multiple alignment track for sequences of 100 vertebrates on UCSC genome browser (Multiz Alignments of 100 Vertebrates).[20] Finally, we looked at the missense substitution analysis algorithm Align GVGD, which assigns an ordered grade ranging from class C0 (most likely neutral) to class C65 (most likely deleterious) based on biophysical distance between aminoacids and evolutionary aminoacid conservation. We considered C45 as a cutoff for a probably deleterious effect.[21] Clinical interpretation of the variants was based on ACMG guidelines.[22] For family 9, recessive (homozygous or compound heterozygous) and de novo inheritance with parental mosaicism were also evaluated.

Variants were confirmed by Sanger sequencing and were investigated by Sanger sequencing in additional relatives with available DNA.

TABLE 1 Clinical data for the cohort of sporadic cases

Clinical data Uterine phenotype	n = 68
Adult cases	65
MRKH type 1	24
MRKH type 2	33
Other	
Uterovaginal aplasia and abnormaldevelopment of secondary sexual characteristics or streak ovaries or gonadal dysgenesis	4
Hemi-uterus	2
Bicorporeal uterus	2
Fetuses	3
Uterovaginal aplasia (non-isolated)	2
Bicorporeal uterus	1
Associated kidney malformation	29/68
Family history of renal/uterine malformations	7/68

Abbreviation: MRKH, Mayer-Rokitansky-Kuster-Hauser syndrome.

2.3 | cDNA synthesis

RNA was extracted from blood collected on a Paxgene tube for individual II:2 in family 9 and an unrelated control. cDNA was synthesized (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit) and then used as template for amplification. PCR was conducted using the following primers for *GREB1L* (NM_001142966.2): Forward (in exon 22)-5'- ACCACGCTGACTATAGCAACCAG -3', Reverse (at the junction between exon 23 and 24)-5'- CACTGCTTTCATTGTGATCGGT -3'. PCR products for the patient and the control were run on a 2.5% agarose gel electrophoresis. The bands were then extracted and Sanger sequenced.

2.4 | Targeted *GREB1L* sequencing

Regions of interest were captured using the single molecule Molecular Inversion Probes (smMIPs) technology.[23] The smMIPs (EasySeqNGS-customized Targeted Capture Kit, Nimagen) were designed to target the exons ±20 nucleotides of intronic/exonic boundaries and the 5'UTR region of *GREB1L* (NM_001142966.2). Massive parallel sequencing on a Nextseq 500 Illumina sequencer generated 150 bp paired-end reads. More than 95% of nucleotides were covered at least ×40 and more than 91% of the targeted region, including the entirety of the exons 2 to 33 (with exception for 56 nucleotides within the exon 22) were covered more than ×100. Exon 1 (non-coding exon) had a lower depth of coverage. The gap in exon 22 (Hg19, chr18:g.19079859 to chr18:g.19079915) was filled in with Sanger sequencing (primers for *GREB1L* (NM_001142966.2): Forward-5'- TGGAGAATGGAGTGAGCTCTTCCA-3', Reverse-5'- TACCTGTGGGGGCCCTGTCA-3′). Single nucleotide variations were interrogated using the Seqnext (SeqPilot) software. A lower threshold of 2% was used for variant allele detection. All rare variants identified by targeted sequencing were confirmed by Sanger sequencing.

3 | RESULTS

In four families, heterozygous deleterious variants were identified in the gene *GREB1L* (Genebank: NM_001142966.2) (Figure 1, Figure 2 and Table 2).

In family 5, WES performed in individuals III:5 and II:1 identified a novel variant consisting in a two base pair deletion (c.2787_2788del) leading to a frameshift (p.(Asp930Profs*12)). Individual III:5 was the mother of a female fetus with bilateral renal agenesis (BRA) and uterovaginal aplasia (DNA not available for this fetus). She was not known to have any kidney or uterine anomalies. Individual II:1, the maternal uncle of III:5, had unilateral renal agenesis (URA). The daughter of II:1 (individual III:2) presented URA and she had a male fetus (IV:1) with URA and contralateral multicystic kidney disease (MCKD). Presence of the variant in individual III:2 and fetus IV:1 was confirmed by Sanger sequencing.

In family 6, WES identified a novel variant, a one base pair deletion (c.2227del) leading to a frameshift and a premature stop codon (p.(Gln743Argfs*10)) in a female fetus (IV:1) with uterovaginal aplasia and bilateral congenital renal anomalies (URA and contralateral MCKD), and individual III:5 (fetus (IV:1)'s mother cousin) affected by MRKH type 2, URA and mild scoliosis. The presence of the frameshift variant was confirmed by Sanger sequencing in III:2 and III:4, respectively, the mother and maternal aunt of fetus IV:1. Individual III:2 was unaffected (renal ultrasound was normal, no uterine malformation reported), whereas individual III:4 had URA and MRKH type 2. Besides the *GREB1L* variant, an heterozygous missense variant (c.2846T>C, p.(Ile949Thr)) was identified in the gene *ROBO2* (NM_001290040.1) in individuals III:2,III:4,III:5 and IV:1. This variant was previously described in one family with CAKUT (vesicoureteral reflux and hypoplastic kidneys).[24] The variant affects a highly conserved nucleotide (GERP++RS Score: 6.16) and a moderately conserved aminoacid. The variant is present in a very low frequency in the population database Gnomad (MAF:0.00001219). However, although a contribution of this variant to the phenotype in family 6 cannot be ruled out, there is no existing functional data for this *ROBO2* variant. In addition, in silico predictions (PolyPhen-2, PROVEAN, SIFT, Mutation assessor, Align GVGD and the CADD score) are rather suggestive of a benign/non-deleterious effect.

In family 7, a novel heterozygous missense variant (c.5198A>G, p.(Asn1733Ser)) was identified by WES in a female fetus (IV:1) with uterovaginal aplasia, BRA, streak gonads, ureter and bladder aplasia, as well as in the sister (IV:2) and in the father (III:1) both presenting URA. Familial analyses by Sanger sequencing identified the variant in one unaffected paternal aunt (individual III:4) (normal renal ultrasound) and the paternal grandmother (II:2) (not known to be affected). The niece of II:2 (individual III:6) had URA and a hemi-uterus but she was not available for testing. The identified missense variant was absent from the population database Gnomad and was predicted to be deleterious or likely deleterious by five in silico programs (PolyPhen-2, PROVEAN, SIFT, Mutation Taster and Mutation assessor). Additional in silico evidences for deleteriousness were the CADD score at 26.1 and the AlignGVGD class at C45. The variant affects a highly

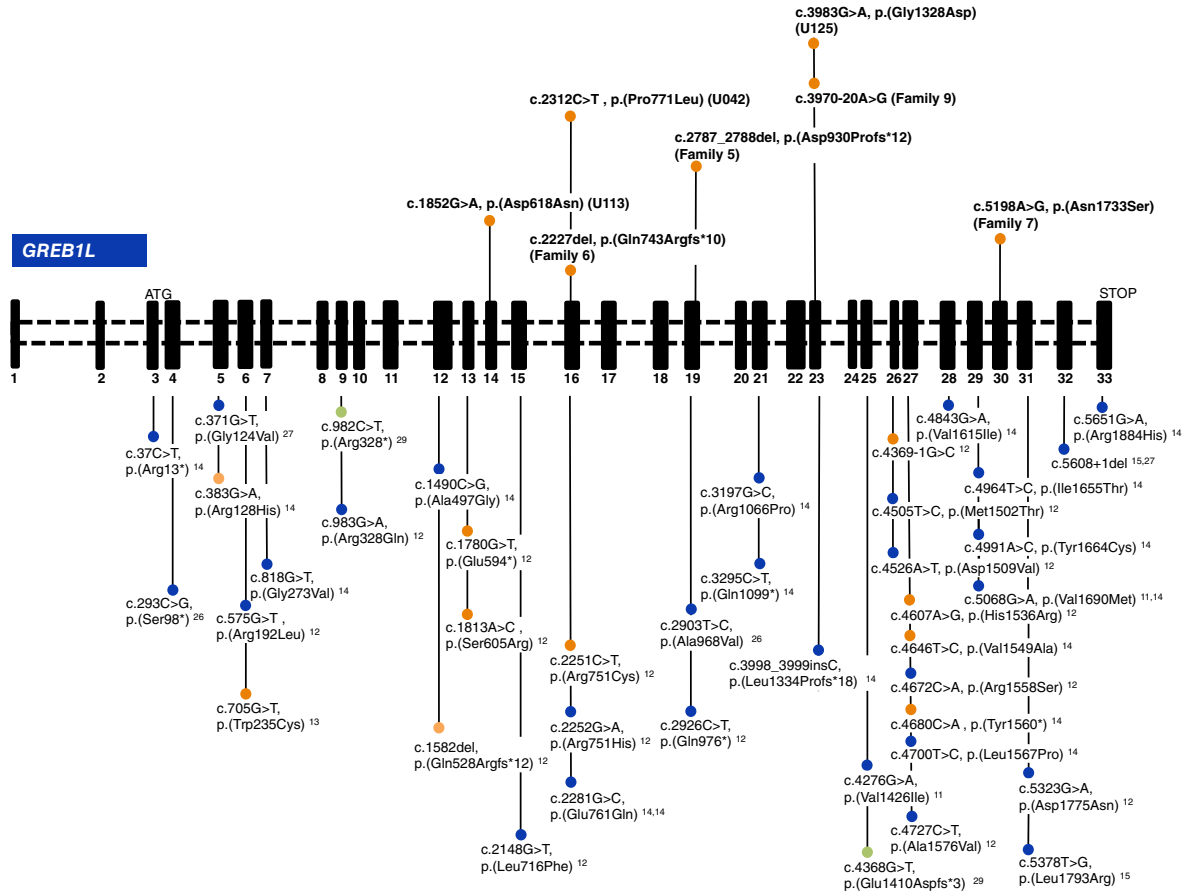

FIGURE 2 Variants in *GREB1L* (NM_001142966.2) reported in the literature. On the top, variants from this publication and underneath, the variants previously reported. Variants associated with congenital uterine (± renal) anomalies are pointed in orange. Variants associated with only congenital renal anomalies are pointed in blue. Variants associated with non-syndromic inner ear malformation are pointed in green

conserved nucleotide (GERP++RS score of 5.89) and a highly conserved amino acid, from zebrafish to human.

In family 9, an intronic variant (c.3970-20A>G), was identified in two fetuses with partial uterovaginal aplasia, BRA, ureter and bladder aplasia (III:1 and III:2) as well as in their asymptomatic father (II:2) (normal renal ultrasound). In addition, the fetus III:2 presented unilateral hexadactyly. This variant was located 20 nucleotides before the boundary between intron 22 and exon 23 and was predicted to create a new acceptor splice site (Human Splicing Finder, Alamut Visual splicing predictions tools).

Amplification of the fragment between exon 22 and exon 24 was performed on cDNA from the father (II:2) and an unrelated control. Variability in amplification of the targeted region was observed, probably due to very low level of *GREB1L* expression in blood (according to GTEx Portal, an open access database on tissue-specific gene expression).[25] For the control, sequencing of the PCR products after extraction of the electrophoretic band confirmed the normal sequence. For the case, aberrant electrophoretic bands were variably detected (Figure 3). Sequencing of the PCR products showed the expected normal sequence as well as an alternative sequence with 19 additional nucleotides between exon 22 and 23 (Figure 3), leading to a frameshit and a premature stop codon (p.(Val1324Leufs*34)). Overlap of the normal and alternative cDNA sequences was observed for the third electrophoretic band. Low *GREB1L* expression in blood as well as random and variable amplification of each DNA strand (the wild type strand and the strand with the variant) might explain why we did not consistently observe the alternative transcript. Moreover, the alternative splicing might not be complete or lead to an unstable mRNA, thus reducing the level of aberrant mRNA in the blood.

Finally, based on the hypothesis that most severely affected individuals would have inherited additional genetic factors from their second parent, the exome data for fetuses in families 7 and 9 were filtered looking for additional rare variants, that is, in family 7, present in the fetus IV:1 but absent in the father III:1 and the sister IV:2, and in family 9, present in both fetuses III:1 and III:2, but absent in the father II:2. We focused on genes previously involved in CAKUT or

TABLE 2 Information on *GREB1L* variants identified in familial and sporadic cases

Identification	Phenotype	NGS	Nucleotide change	Aminoacid change	State	Conservation GERP++RS score	PP2 score/ SIFT/ MT	Align GVGD	Scaled CADD score	Splicing in silico prediction (HSF)	GnomadMAF (Hm/Hz/ total)	ACMG classification
Variants of clinical interest												
Family 5		WES	c.2787_2788del	p.(Asp930Profs*12)	Hz	5.71	−/−/D.C	—	—	—	NR	Pathogenic
IV: 1[a]	URA and MCKD											
III: 2	URA											
II: 1	URA											
III: 5	Asymptomatic (fetus with uterovaginal aplasia and BRA)											
Family 6		WES	c.2227del	p.(Gln743Argfs*10)	Hz	6.08	−/−/D.C	—	—	—	NR	Pathogenic
IV: 1[a]	URA and MCKD											
III: 5	MRKH type 2,URA,mild scoliosis											
III: 4	MRKH type 2,URA											
Family 7		WES	c.5198A > G	p.(Asn1733Ser)	Hz	5.89	0.994/D./ D.C.	C45	26.1	No significant alteration	NR	Uncertain significance
III: 1	URA											
IV: 2	URA											
IV: 1[a]	Uterovaginal aplasia,BRA, streak gonads,ureter and bladder aplasia											
Family 9		WES	c.3970-20A > G	p.(Val1324Leufs*34)	Hz	0 (na)	−/−/Pm	—	0.490	New acceptor site	NR	Likely pathogenic
III: 1[a]	Uterovaginal aplasia,BRA Ureter and bladder aplasia											
III: 2[a]	Uterovaginal aplasia,BRA Ureter and bladder aplasia Unilateral hexadactyly											
II: 2	Asymptomatic											
U113	Ectopic kidney VUR Duplicated ureter MRKH type 2 Unilateral polydactyly Facial asymetry	smMIPS	c.1852G > A	p.(Asp618Asn)	Hz	5.68	0.998/ T/D.C.	C0	25.1	No significant alteration	0.00002108 (0/4/189764)	Uncertain significance
U042[a]	Uterovaginal aplasia Streak ovaries URA-MCKD 11 pairs of ribs Cervical hemivertebrae	smMIPS	c.2312C > T	p.(Pro771Leu)	Hz	6.08	0.999/D/ D.C.	C65	31	No significant alteration	0.00003187 (0/1/31378)	Uncertain significance

(Continues)

TABLE 2 (Continued)

Identification	Phenotype	NGS	Nucleotide change	Aminoacid change	State	Conservation GERP++RS score	PP2 score/ SIFT/ MT	Align GVGD	Scaled CADD score	Splicing in silico prediction (HSF)	GnomadMAF (Hm/Hz/ total)	ACMG classification
U125	MRKH type 2 URA	smMIPS	c.3983G > A	p.(Gly1328Asp)	Hz	4.68	1/D/ D.C.	C65	27,4	No significant alteration	NR	Uncertain significance
Other variants												
U098	MRKH type 1	smMIPS	c.1936 T > C	p.(Cys646Arg)	Hz	4.67	0.001/ T/D.C	C0	20.9	Alt. exonic ESE site	NR	Uncertain significance
U147	MRKH type 2 Ectopic kidney	smMIPS	c.3492G > T	p.(Gly1164=)	Hz	0 (na)	–/T/Pm	–	0.028	Alt. exonic ESE site	NR	Uncertain significance
U120	MRKH type 1	smMIPS	c.277G > A	p.(Glu93Lys)	Hz	5.35	0.291/D/ D.C.	C0	24.6	Alt. exonic ESE site	0.002813 (1/ 532/ 189090)	Uncertain significance
U149	MRKH type 2 Mild caliectasis Vertebral anomalies Imperforate anus	smMIPS	c.277G > A	p.(Glu93Lys)	Hz	5.35	0.291/D/ D.C.	C0	24.6	Alt. exonic ESE site	0.002813 (1/ 532/ 189090)	Uncertain significance
Family 9 (II: 2;III: 1[a]; III:2[a])	Phenotype described above	smMIPS	c.277G > A	p.(Glu93Lys)	Hz	5.35	0.291/D/ D.C.	C0	24.6	Alt. exonic ESE site	0.002813 (1/ 532/ 189090)	Uncertain significance

Notes: Splicing prediction: at least three algorithms for ESE (Exonic Splicing Enhancer)/ESS (Exonic Splicing Silencer) alteration on Human Splicing Finder; at least two algorithms for new donor/acceptor site. PolyPhen-2, SIFT, Mutation Taster, CADD, Align GVGD (v2007), and theGERP++ RS score were used for prediction.

Abbreviations: BRA, bilateral renal agenesis; D, damaging; D.C., disease causing; MCKD, multicystic kidney disease; MRKH, Mayer-Rokitansky-Kuster-Hauser syndrome; na, data not available; NR, not reported; Pm, polymorphism; T, tolerated; URA, unilateral renal agenesis; WES, whole exome sequencing.

[a]Fetus.

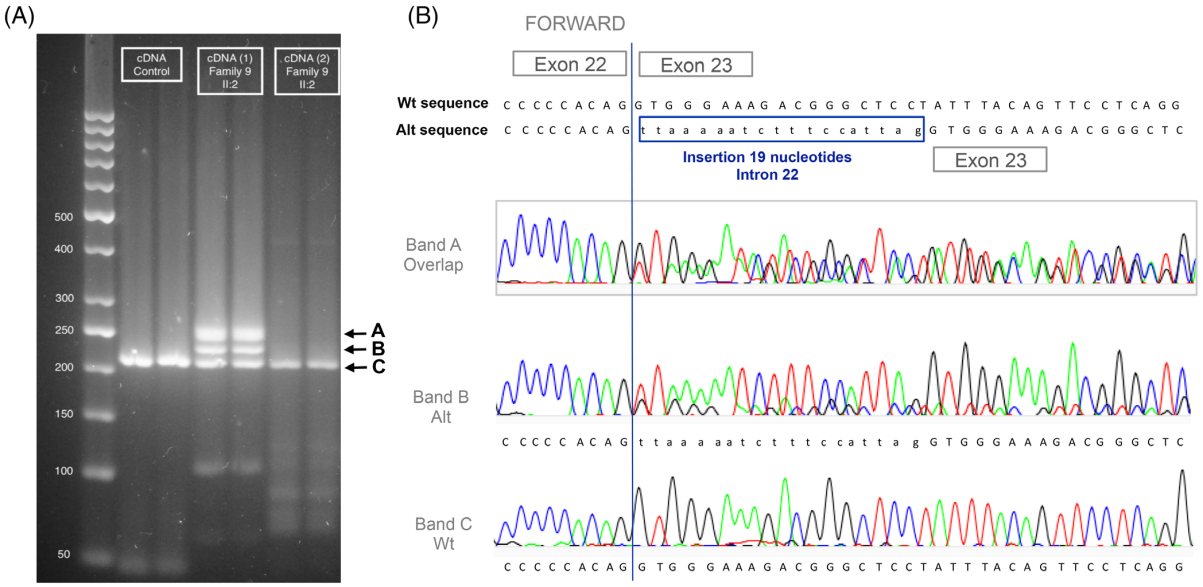

FIGURE 3 Results of agarose gel electrophoresis of cDNA (family 9 II:2) A, *GREB1L* PCR products and B, sequence analysis for the novel potential splice-site mutation c.3970-20A>G. Three bands (around 250 bp [band A], at 229 bp [band B] and at 210 bp [band C]) were discernible on agarose gel electrophoresis. Sequence analyses demonstrated that band C was the sequence from the wild type transcript and that the alternative sequence (band B) resulted from insertion of the last 19 bp of intron 22. Sequencing of band A showed an overlap between both wild type and alternative sequences

MRKH, in the GREB1/ER interactome or in the Wnt pathway. These variants are described in Table S4. No likely pathogenic or pathogenic variant could be identified.

Targeted sequencing of *GREB1L* in a cohort of 68 unrelated individuals with uterine malformations (including three fetuses) is presented in Table 1, led to identify rare variants of unknown significance (four missense and one synonymous) in three individuals with MRKH type 2 (U113, U125, U147), one fetus with uterovaginal aplasia and bilateral kidney malformation (U042), and one individual with MRKH type 1 (U098) (Table 2). For most individuals, parental DNA was not available to check for de novo occurrence. The variant identified in fetus U042 was inherited from the asymptomatic father (normal renal ultrasound). Finally, the variant (c.277G>A, p.(Glu93Lys)) (rs185578147)was identified in two additional unrelated individuals (U120 and U149) as well as in family 9 (II:2; III:1;III:2). Considering the entire cohort (9 familial cases and 68 sporadic cases [3 of 77]), the allele frequency for this variant was 0.019 (3 of 154) which was 3.85 times higher than the minor allele frequency reported in Gnomad (MAF: 0.004939 in European [non-Finnish] population). At present, the association between this variant and the phenotype cannot be asserted. Finally, we did not identify any rare mosaic variant considering a minimum allele frequency of 2%.

4 | DISCUSSION

GREB1L disease-causing variants have been previously reported in families and sporadic cases of congenital anomalies of kidneys and optionally of the uterus.[11-15,26,27] Haploinsufficiency is the suggested mechanism for pathogenicity[15] with 10 loss-of-function variants out of the 39 variants reported thus far. CUA were noted in 10 of these 41 families and sporadic cases.[12-14] We describe four additional familial cases and three sporadic cases with uterovaginal aplasia or MRKH syndrome type 2 and convincing *GREB1L* variants.

Among the previously reported *GREB1L* families and herein, formation defects were the most frequently observed developmental defect of the uterus, with aplastic uterus or MRKH type 2 being reported in 15 individuals with *GREB1L* convincing variants and hemiuterus in three. Fusion and septal absorption defects were uncommon with bicorporeal and arcuate uterus each described in one individual (Table 3). Additional anomalies of the female genital tract and ovaries included in some cases agenesis of the fallopian tubes and ovaries, streak gonads, hemi-vagina and absence of the uterine left artery (Table 3). The CAKUT phenotypes were most of the time renal agenesis, renal dysplasia/hypoplasia, vesicoureteral reflux and MCKD (Table 3). Bladder/ureter agenesis/hypoplasia were described in six fetuses with BRA. Megaureter, duplication of the ureters and ectopic kidney were each reported in two cases. In individuals with MRKH or uterovaginal aplasia, renal agenesis (uni or bilateral) or MCKD were consistently associated, whereas, with the other types of uterine anomalies, kidney anomalies were not always present. Conversely, uterine anomalies were not always noted in females in case of renal anomalies.

In our cohort, we also identified one novel missense variant (c.1936T>C, p.(Cys646Arg)), not reported in Gnomad, in one individual with MRKH type 1 (U098). However, conflicting in silico

TABLE 3 Phenotypic features in heterozygous carriers of convincing *GREB1L* variants

Phenotype	Our study (20 individuals [from 7 families])	Literature (96 individuals [from 54 families])	Total (116 individuals [from 61 families])
Renal			
Unilateral renal agenesis	10/20	28/96[11-15,27]	38/116
Bilateral renal agenesis	3/20	27/96[12-15,26,27]	30/116
Multicystic kidney disease	3/20	7/96[12-14]	10/116
Vesicoureteral reflux	1/20	7/96[11,12,14]	8/116
Renal hypoplasia/dysplasia		7/96[11,12,14]	7/116
Bladder hypo/aplasia	3/20	3/96[15,27]	6/116
Ectopic kidney	1/20	1/96[12]	2/116
Duplication of the ureter	1/20	1/96[14]	2/116
Megaureter		2/96[14]	2/116
Megaurethra		1/96[12]	1/116
Horseshoe kidney		1/96[12]	1/116
Multilocular cyst		1/96[12]	1/116
Clear cell renal carcinoma		1/96[13]	1/116
Genital			
Uterus/uterovaginal aplasia	4/20	5/96[12,14]	9/116
MRKH type 2	4/20	2/96[13]	6/116
Hemi-uterus		3/96[12,14]	3/116
Streak gonads	2/20		2/116
Unique follapian tube and/or ovary		2/96[12,14]	2/116
Uterus anomaly (not described)		1/96[14]	1/116
Blind ending hemi-vagina and bicornuated uterus		1/96[12]	1/116
Arcuate uterus		1/96[13]	1/116
Absence of uterine left artery		1/96[12]	1/116
Ovarian hernia		1/96[12]	1/116
Undifferentiated external genitalia		1/96[15,27]	1/116
Heart			
Thickened left ventricular wall		2/96[12]	2/116
Aortic stenosis		1/96[12]	1/116
Retro-esophageal subclavian artery		1/96[12]	1/116
Adrenal			
Adrenal gland hypoplasia		1/96[12]	1/116
Adrenal cytomegaly		1/96[12]	1/116
Liver			
Hepatic portal fibrosis		1/96[12]	1/116
Skeletal anomalies			
Ribs anomalies	1/20	3/96[12]	4/116
Cervical hemivertebrae	1/20		1/116
Mild scoliosis	1/20		1/116
Anomalies of the extremities			
Polydactyly	2/20		2/116
Clinodactyly		2/96[12]	2/116
Single transverse palmar crease		1/96[11]	1/116

TABLE 3 (Continued)

Phenotype	Our study (20 individuals [from 7 families])	Literature (96 individuals [from 54 families])	Total (116 individuals [from 61 families])
Brachydactyly		1/96[11]	1/116
Genu valga		1/96[14]	1/116
Flat feet		1/96[14]	1/116
Face			
Facial dysmorphism		1/96[11]	1/116
Facial asymetry	1/20		1/116
Short neck		1/96[11]	1/116
Ear			
Neurosensorial hypoacusia		3/96[14,29]	3/116
Auricular tag		2/96[12]	2/116
Congenital anomalies of the inner ears		2/96[29]	2/116
Absent eighth nerve		2/96[29]	2/116
Lop ear		1/96[12]	1/116
Eye			
Iris anomaly		1/96[12]	1/116
Other			
Enlarged thymus		1/96[12]	1/116
Henoch Schonlein Purpura		1/96[14]	1/116
Diabetes		1/96[12]	1/116
Supernumerary nipple		1/96[11]	1/116
No phenotype			
Asymptomatic (Normal renal US, no known uterine anomaly)	4/20	9/96[12,13,15,27]	13/116
Not ascertained by renal US	1/20	7/96[12,14]	8/116

predictions make this variant less likely to be causative, most in silico tools (PolyPhen-2, PROVEAN, SIFT, Mutation assessor, CADD, Align GVGD) supporting a neutral/non-deleterious effect, whereas, only Mutation Taster and Human Splicing Finder states for a possible damaging effect (Table 2). If *GREB1L* seems to contribute to isolated CUA in some individuals, sequencing in additional cohorts of MRKH individuals with accurate clinical examination would be necessary to determine its possible involvement in MRKH type 1.

Variability in the phenotype among our patients across or within families could not be solely explained by the type or the location of *GREB1L* variants. These were scattered throughout the gene and included loss-of-function as well as deleterious missense mutations in both individuals with isolated renal anomalies or renal and uterine anomalies, with no hotspot for uterine malformation (Figure 2). The incomplete penetrance and intrafamilial variability observed in our study, and previously reported,[27,28] may result either from stochastic differences during development, the influence of modifier genes or additional variants in *cis* or *trans*, variation in allele dosage, epigenetic factors or environmental exposures.

Maternal bias in transmission was previously suggested as it was observed that the mother was the transmitting parent in most families.[12,13]In our four families, we did not confirm parental bias and could not explain non-penetrance by the gender of the transmitting parent. Among the affected individual carriers of *GREB1L* variants for whom the status of the parents was known (by genotyping or because they were obligate carriers), five individuals inherited the variant from their mother and five from their father. The severity of the phenotype was not correlated with the gender of the transmitting parent. Indeed, for the five affected fetuses with BRA (or URA+MCKD) ± uterovaginal aplasia, two inherited the variant from their mother and three from their father. Finally, three unaffected females inherited the variant from their mother. In families 5, 6 and 7, a three-generation pedigree indicated a more severe phenotype for fetuses at the third generation, as also previously reported in a single family.[13] This is probably due to ascertainment bias rather than anticipation as families are sooner referred to geneticists in case of severe and lethal congenital anomalies. Also, pregnancy losses in prior generation may not have been assigned to BRA.

In *GREB1L* families and sporadic cases with congenital anomalies of kidneys and uterus, additional clinical features were sometimes present, expanding the clinical spectrum. Rib anomalies, heart defects, anomalies of the adrenal glands, polydactyly and ear anomalies were reported in more than one individual, and some other only once (Table 3). Moreover, in a study on non-syndromic inner ear malformation, two de novo loss-of-function *GREB1L* variants were identified in two male individuals with sensorineural hearing loss, absent cochleae and eighth cranial nerve malformations without any known kidney anomaly.[29]Abnormal sensory epithelial innervation was demonstrated in zebrafish for the *greb1l* homozygous mutant sa16374.[29] As both variants were loss-of-function, haploinsufficiency was also suggested as the mechanism of pathogenicity. Interestingly, hearing loss is reported in 10% to 25% of individuals with MRKH syndrome.[30] In this series, no anomalies of the auditory system were reported in individuals with *GREB1L* variant, but hearing tests were not systematically performed.

Among the nine families investigated herein, plausible *GREB1L* variants were detected in 4 of 9 (44%), and the gene is currently the most frequent one associated with familial congenital uterine malformations. *GREB1L* sequencing in all reported CUA/MRKH families would help to better define the frequency in families with recurrence. All our families with *GREB1L* variants included individuals with MRKH type 2/uterovaginal aplasia and fetuses with bilateral renal anomalies (renal agenesis or MCKD). The diagnostic yield for *GREB1L* mutation was previously shown to be around 0.86% in isolated CAKUT (2/232),[11] 2.8% in renal agenesis or renal hypodysplasia[14] and much higher (25.8%) in fetuses with BRA.[12] The presence of uterine malformation or MRKH might thus increase even more significantly the chance to find *GREB1L* mutation. We therefore suggest investigating this gene in all families with uterovaginal aplasia or MRKH syndrome, with renal agenesis.

This study is the first to interrogate the presence of *GREB1L* mutations in sporadic MRKH/uterovaginal aplasia. In our cohort of 63 individuals, *GREB1L* heterozygous variants were identified in 7.9% (5 of 63) with most convincing variants in three (4.8%), all with a MRKH type 2/non-isolated uterovaginal aplasia phenotype. This suggests that *GREB1L* might also be a significant genetic contributor in sporadic MRKH/uterovaginal aplasia, at least when renal anomalies are present (MRKH type 2). The presence of somatic mosaicism to explain sporadic cases was not supported by our results. Analysis of affected tissues (ie, rudimentary uterine tissue) would, although, be more appropriate to investigate this hypothesis. In addition, the apparent phenotypic continuum of CUA and MRKH syndrome associated with various malformations raises the possibility of a digenic/oligogenic origin, which could help understand the complexity of the disease and the still unexplained cases.

ACKNOWLEDGEMENTS

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ETHICS STATEMENT

The genetic alterations in rare diseases protocol was approved by the UBC Children's and Women's Research Ethics Board (Vancouver, Canada). The genetic alteration in congenital uterine anomalies (identification of genetic factors involved in uterine development by exome sequencing of individuals with hereditary or syndromic uterine and kidney malformation) protocol was approved by the ethics committee from the University Hospital and the University of Liège, Belgium. The Programme de Recherches sur les Aplasies Müllériennes project was approved by the local Institutional Review Board (Comité de Protection des Personnes), and is registered with the French Ministry of Health. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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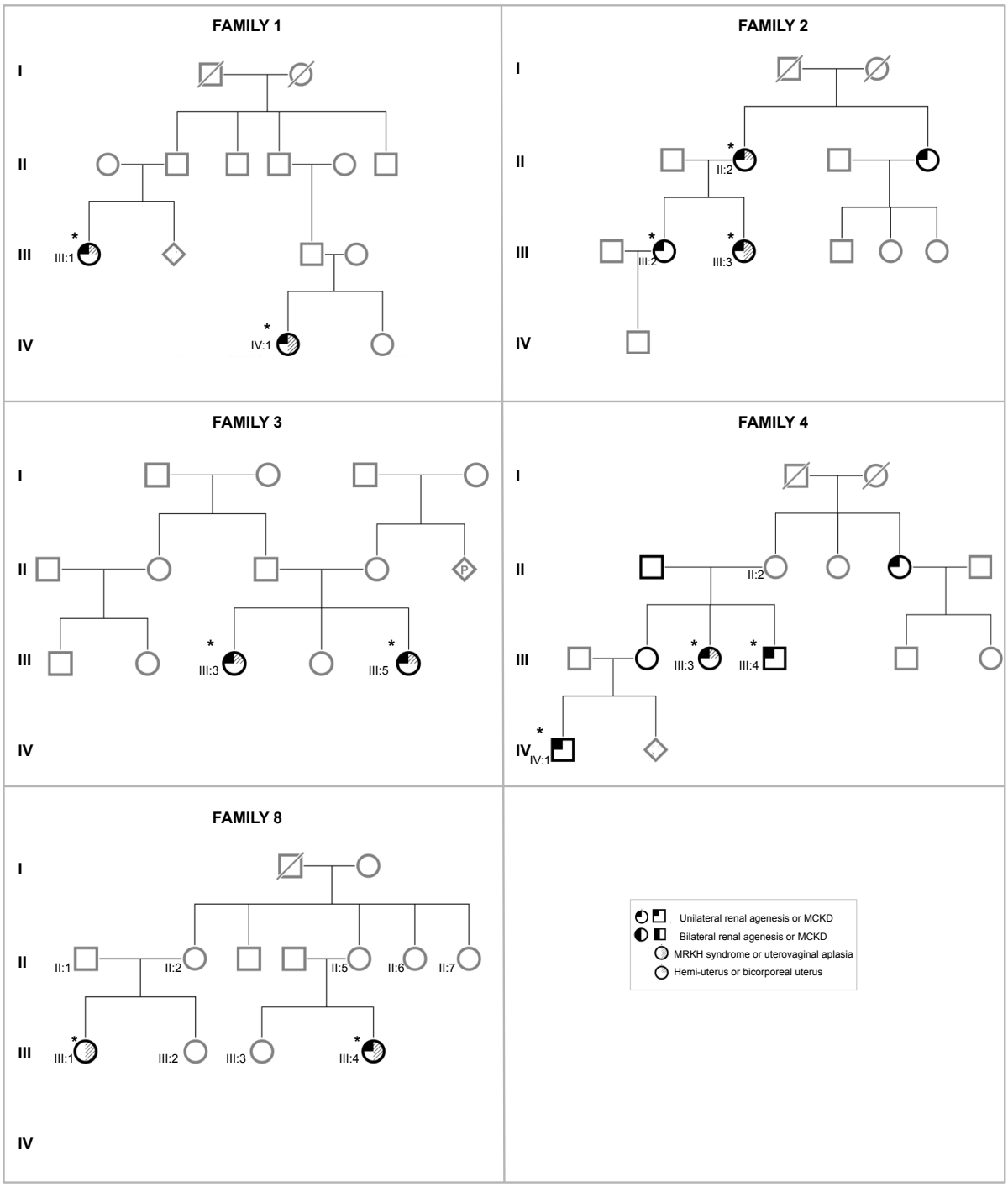

Figure S1 Pedigree of the five families without any identified variants in *GREB1L*. Male and female symbols are black when the phenotype was known for the individual (renal US performed, no known uterine malformation). Male and female symbols are gray when the individual's phenotype was unknown. An asterisk indicates the relatives in whom WES was performed. Symbol numbers indicates individuals with available DNA. Pedigrees were generated using PedigreeXP (http://www.pedigreexp.com).

Supplemental data -Table 1: Phenotype of affected relatives in the nine families

FAMILY	INDIVIDUAL	UTERINE ANOMALY	RENAL ANOMALY	OTHER
Family 1	**IV :1**	MRKH type 2	Unilateral MCKD	
	III :1	Bicorporeal uterus	Unilateral renal agenesis	Scoliosis
Family 2	**III :3**	MRKH type 2	Unilateral renal agenesis, ectopic kidney, VUR	
	III :2	Normal	Right renal hypoplasia	
	II :2	Hemi-uterus	Unilateral renal agenesis, ectopic kidney	Left hemilombalisation of S1
	II :4	NR	Horseshoe kidney	
Family 3	**III :3**	Complete bicorporeal uterus with a double cervix	Unilateral renal agenesis (R)	Mild S-shaped thoracolumbar curvature with no osseous abnormality seen.
	III :5	Complete bicorporeal uterus with a double cervix	Unilateral renal agenesis (L)	
Family 4	**III :3**	Right hemi-uterus	Unilateral renal agenesis	Policystic ovary disease
	IV :1	/	Unilateral renal agenesis	Learning disability (processing disorder)
	III :4	/	Unilateral renal agenesis	Unusual configuration of the liver (the left lobe being nearly as large as the right), Single testis
Family 5	**IV :1 (fetus 23w)**	/	Unilateral renal agenesis and MCKD	
	III :2		Unilateral renal agenesis	
	II :1		Unilateral renal agenesis	
	III :5	/	/	Had a fœtus with uterovaginal aplasia and bilateral renal agenesis
Family 6	**IV :1 (fetus 23w)**	Uterovaginal aplasia	Unilateral renal agenesis and controlateral MCKD	
	III :5	MRKH type 2	Unilateral renal agenesis	Mild scoliosis
	III :4	MRKH type 2	Unilateral renal agenesis	
Family 7	**III :1**	/	Unilateral renal agenesis	
	IV :2	/	Unilateral renal agenesis	
	IV :1 (fetus 18w)	Uterovaginal aplasia	Bilateral renal agenesis	Streak gonads, ureter and bladder aplasia
	III :6	Hemi-uterus	Unilateral renal agenesis	
Family 8	**III :1**	MRKH type 1	/	
	III :4	MRKH type 2	Unilateral renal agenesis	
Family 9	**III :1 (fetus 16w)**	Uterovaginal aplasia	Bilateral renal agenesis	Ureter and bladder aplasia
	III :2 (fetus 18w)	Uterovaginal aplasia	Bilateral renal agenesis	Ureter and bladder aplasia, unilateral hexadactyly

Supplemental data -Table 2: Phenotype of sporadic cases

ID	Genital	Kidney	Other	Family History
U042#	Uterovaginal aplasia, rudimentary follopian tubes, streak gonads	Right renal agenesis and left multicystic kidney disease	Right unique umbilical artery, 11 ribs pairs, 6 cervical vertebrae with 1 hemivertebrae	
U045	MRKH type 1	NR	Craniopharyngiome, constipation	
U048	MRKH type 2	Right renal agenesis, left VUR	Mild scoliosis, autoimmune thyroidite, absence of widening of the interpedicular distance	
U051	MRKH type 2	Unilateral renal agenesis	Scoliosis, 11 ribs pairs, micrognathia, pelvis asymmetry	
U058°	Bicorporeal uterus	NR	Hemivertebrae D6-D7 and L2-L3; 13 ribs on the right	
U061	MRKH type 2	NR	Scoliosis	
U062	MRKH type 2	NR	Cyphoscoliosis, L5 sacralization	
U064	MRKH type 2	NR	Scoliosis, hearing loss	
U066	MRKH type 1	NR	/	Unilateral renal agenesis (uncle)
U067	Uterovaginal aplasia, streak gonads	Unilateral renal agenesis	Hypoparathyroidism (familial)	Unilateral renal agenesis (uncle)
U068	MRKH type 1	NR	Cervical hyperlordosis	
U070	MRKH type 1	NR	/	Unilateral renal agenesis (son of the father's cousin); bilateral ectopic testis (brother and maternal uncle)
U071	MRKH type 2	Unilateral renal agenesis	/	
U072	MRKH type 2	NR	L5 sacralization, 11 ribs pairs	
U073	MRKH type 2	NR	Atrial septal defect	
U074	MRKH type 1	NR	/	
U075	MRKH type 1	NR	/	
U077#	Bicorporeal uterus	Bilateral renal agenesis	/	Bicorporeal uterus and unilateral renal agenesis (mother)
U079	MRKH type 2	Unilateral renal agenesis	/	
U081	MRKH type 1	NR	/	
U082	MRKH type 2	NR	Mild scoliosis	Scoliosis (sister), Unilateral renal agenesis (maternal cousin)
U083	MRKH type 2	Unilateral renal agenesis	/	
U085	MRKH type 2	Left ectopic kidney and VUR	/	
U086	MRKH type 2	Left ectopic kidney and VUR	/	
U087	MRKH type 1	NR	/	
U091	MRKH type 1	NR	/	
U092	MRKH type 1	NR	/	

ID	Genital	Kidney	Other	Family History
U093	Hemi-uterus, agenesis of the left ovary	Unilateral renal agenesis	Klippel-Feil syndrome	Bicorporeal uterus and unilateral renal agenesis (paternal aunt)
U094	MRKH type 2	Right renal hypoplasia	Spina bifida	
U095	MRKH type 2	NR	Scoliosis, mild hearing loss	Scoliosis and hearing loss (mother)
U096	MRKH type 1	NR	/	
U098	MRKH type 1	NR	/	
U099	Uterovaginal aplasia	NR	Abnormal development of secondary sexual characteristics, mitral valve prolapsus, arachnodactyly (associated Marfan syndrome)	
U100	MRKH type 2	Unilateral renal agenesis	/	
U104	MRKH type 2	Horseshoe kidney, left pelvis triplication	Atrial septal defect, dysmorphic facial features (associated 2q37 deletion)	
U105	MRKH type 1	NR	/	
U106	MRKH type 2	Unilateral renal agenesis	/	
U107	MRKH type 2	Ectopic horseshoe kidney	C3-C5 fusion, scoliosis	C3-C5 fusion (paternal grand-father)
U108	MRKH type 2	NR	Scoliosis	
U109	MRKH type 2	NR	Scoliosis	Isolated atrial septal defect (brother)
U110	MRKH type 1	NR	/	
U111#	Uterovaginal aplasia	Bilateral renal agenesis	Spina bifida	
U112	MRKH type 1	NR	/	
U113	MRKH type 2	Ectopic kidney, VUR, duplicated ureter	Left hand polydactyly, facial asymetry	
U115	MRKH type 2	Unilateral renal agenesis	Mild scoliosis	Unilateral renal agenesis (maternal cousin)
U116	Uterovaginal aplasia, streak or absent gonads	NR	Abnormal development of secondary sexual characteristics	
U117	MRKH type 1	NR	/	
U118	MRKH type 1	NR	/	
U119	MRKH type 2	Bilateral renal hypoplasia	/	
U120	MRKH type 1	NR	/	
U121	MRKH type 1	NR	/	
U122	MRKH type 1	NR	Elevated LH and testosterone	

ID	Genital	Kidney	Other	Family History
U123	MRKH type 2	Normal	Scoliosis , D3 hemivertebrae, unilateral thumb aplasia and hand fixed in flexion, oesophageal atresia, tracheomalacia, dextrocardia with a right dilated ventricule	
U124	MRKH type 2	Unilateral renal agenesis	/ (associated 1q21.1 duplication (Hg19, 1:144530059-146290831)	
U125	MRKH type 2	Unilateral renal agenesis	/	
U126	MRKH type 1	NR	/	
U127	Right hemi-uterus, aplastic vagina	Left ectopic and hypoplastic kidney	Ventricular septal defect with pulmonary hypertension	
U129	MRKH type 1	NR	/	
U130	MRKH type 2	Bilateral duplicated ureters	/	
U131	MRKH type 2	Unilateral renal agenesis	Imperforate anus, Klippel-Feil syndrome	
U132	MRKH type 2	NR	Vertebral malformation (unspecified)	
U133	MRKH type 1	NR	Type 1 diabetes	
U144	Uterovaginal aplasia, ovarian dysgenesis	NR	Klippel-Feil syndrome, hearing loss, abnormal aortic valve	
U145	MRKH type 2	Ectopic right kidney	/	
U146	MRKH type 1	NR	/	
U147	MRKH type 2	Ectopic kidney	/	
U148	Bicorporeal uterus	Unilateral renal agenesis	/	
U149°	MRKH type 2	Mild caliectasis	Imperforate anus, D11-D12 fusion and D12-L1, L1-L2, L3-L4 partial fusion	

[#] fetus, ° individual included through the GACUA protocol, NR : not reported

Supplemental data- Table 3: In silico tools used to interprete the variants of interest

In silico tool	Web link/software consulted
PolyPhen-2	http://genetics.bwh.harvard.edu/pph2/
SIFT	http://provean.jcvi.org/protein_batch_submit.php?species=human
PROVEAN	http://provean.jcvi.org/protein_batch_submit.php?species=human
Mutation Taster	http://www.mutationtaster.org
Mutation Assessor	http://mutationassessor.org/r3/
Human Splicing Finder	http://www.umd.be/HSF/
Alamut Visual	Interactive biosoftware (SOPHIAGENETICS.com)
CADD	https://cadd.gs.washington.edu/snv
GERP++RS score	https://genome.ucsc.edu
Multiz Alignments of 100 Vertebrates	https://genome.ucsc.edu
Align GVGD	https://agvgd.iarc.fr

Supplemental data- Table 4: Additional variants identified in fetuses in families 7 and 9 (not inherited from the same parental carrier of the *GREB1L* variant), in genes previously reported in CAKUT or CUA, in genes of the ER/GREB1 interactome or in genes of the Wnt Pathway

	Location	Nucleotide change	Aminoacid change	State	MAF (Gnomad)	PP2 score SIFT MT	ACMG Interpretation (Varsome)	dbSNP
Family 7								
PKHD1 **NM_138694.4**	6-51934318-C-T	c.715G>A	p.(Val239Ile)	Hz	NR	0.026/T/Pm	Uncertain significance	/
MYH11 **NM_001040114.1**	16-15811130-C-T	c.5392G>A	p.(Glu1798Lys)	Hz	0.00001991	0.969/D/D.C.	Likely Benign	rs368269107
AGO3 **NM_024852.4**	1-36505523-C-T	c.1975C>T	p.(Arg659Trp)	Hz	NR	0.978/D/D.C.	Uncertain significance	/
Family 9								
HOXA7 **NM_006896.4**	7-27196213-C-T	c.-49G>A	/	Hz	0.00002682	/	Uncertain significance	rs374752599
PTK7 **NM_001270398.1**	6-43098374-G-A	c.811G>A	p.(Glu271Lys)	Hz	0.000007072	0.027/T/D.C. (Revel : Benign (Moderate) (0.06))	Uncertain significance	rs771338072
SALL1 **NM_002968.2:**	16-51175655-C-T	c.478G>A	p.(Gly160Ser)	Hz	0.0008708	0.000 /T/Pm	Benign	rs113614842

II. *NR6A1*, A NEW GENE INVOLVED IN CONGENITAL ANOMALIES OF THE KIDNEY, UTERUS AND VERTEBRAE IN HUMANS

1. SUMMARY

In cases where no compelling variants were identified in known genes, our secondary objective was to pinpoint potential new candidate genes and confirm their involvement in kidney and uterus development through functional analyses and animal models. In one family (Family 2 of the total cohort, but referred as Family 1 in this manuscript), a heterozygous missense variant in the *NR6A1* gene, shared by three affected relatives, was selected for further investigation. This variant was predicted to be deleterious and was not previously registered in population databases. Existing studies using animal models demonstrated that *NR6A1* is essential for embryonic development, with mice deficient in this gene showing early lethality and posterior truncation defects.

Given the availability of a specialized facility at the GIGA-ULiège and the cost-effectiveness of zebrafish disease modelling, we chose the zebrafish model to initially investigate the role of our candidate gene in embryonic development. Collaboration with Dr Hélène Pendeville, leading manager of the GIGA zebrafish platform, started in 2018. Investigation of the role of *nr6a1* started with morpholino analyses, followed by analysis of the ENU mutant sa34517 (*nr6a1a*). The transgenic line wt1b:GFP was obtained to visualize the pronephros. Following difficulties in obtaining reliable results with the mutant (possibly explained by additional mutations in the background), we decided to generate CRISPR-Cas9 mutants for the two NR6A1 orthologues, *nr6a1a* and *nr6a1b,* in 2020. After observation of differences in the pronephros between mutants and controls, collaboration with the team of Bernard Peers in the ZDDM research lab was started in 2021 to investigate the mutant embryos by *in situ* hybridization, especially looking for patterning defects in the pronephros. In 2022, the adult phenotype in the surviving adults was investigated by microCT scan. At the end of 2022, the paper of Chang et al. was published revealing the role of Nr6a1 as mastor regulator of vertebrate trunk development. Vertebral anomalies were observed in our zebrafish mutant, similar to those previously described in mice. Reverse phenotyping in the familial case revealed mild but significant vertebral and costal anomalies in the most affected relative, fitting the observation in zebrafish and mice. Beside Bernard Peers in the ZDDM team, Lydie Flasse (Postdoc), Manon Dohet (currently a PhD student) and Romane Vanhaeren (currently PhD student), contributed to the study, conducting *in situ* analyses, gel shift assays, and

mesonephros analysis. MicroCT scanning was performed at the CARPOR Platform in collaboration with Erwan Plougonven and Alexandre Leonard.

With this second paper, our main findings regarding *NR6A1* and its characterization in zebrafish will be submitted for publication. Additional experiments and some preliminary results that are not reported in the paper are summarized in the last section.

Variants in *NR6A1* as a cause for congenital renal, vertebral and uterine anomalies

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§ Vincent Bours and Bernard Peers share equal responsibility for the scientific supervision of the research.

ABSTRACT

The underlying cause for renal and uterine agenesis still remains unknown in many cases, whereas recurrence in some families clearly suggests the involvement of genetic factors. In order to uncover novel candidate genes, we performed whole-exome sequencing in three relatives with kidney agenesis/hypoplasia, congenital uterine and costovertebral anomalies, identifying one rare heterozygous missense variant in the orphan nuclear receptor *NR6A1*. Through collaboration and literature review, we identified two additional fetuses with bilateral renal agenesis and a *de novo* heterozygous truncating variant in the gene, and one individual with Mayer-Rokitansky-Küster-Hauser syndrome type 2 and a heterozygous missense variant. *In vitro* studies demonstrated partial loss-of-function for both missense variants. To further investigate the role of *NR6A1* in embryonic development, we generated a knockout model for both orthologues (*nr6a1a* and *nr6a1b*) in zebrafish using CRISPR-Cas9. The mutants exhibited impaired kidney development with anomalies in pronephros segmentation, which translated in hypoplasia of kidney tissues in adults. Defects in the axial skeleton and fins as well as cloacal anomalies were also observed. Finally, we demonstrated that *nr6a1a/b* depletion disrupts the anteroposterior expression pattern of posterior hox genes in zebrafish. Our findings in both humans and zebrafish provide the first evidence of a causal link between *NR6A1* heterozygous loss-of-function variants and renal and costovertebral defects in humans. Furthermore, the phenotype observed in women suggest that uterine malformations may be part of the clinical spectrum.

INTRODUCTION

The association of congenital anomalies in kidneys, uterus and the axial skeleton (vertebrae and ribs) occur more frequently than would be expected based on their respective prevalence. Unilateral renal agenesis is diagnosed in 6.5 to 11.9% of individuals with costovertebral malformations, versus 0.04% in the general population (1,2). Renal and costovertebral anomalies, including scoliosis, are described respectively, in 20% (+/-9%) and 26% (+/-5%) of individuals with Mayer-Rokitansky-Küster-Hauser syndrome (3–5). Their non-random co-occurrence is also highlighted by the delineation of specific clinical entities such as VACTERL (vertebral defects (V), anorectal malformation (A), cardiac anomalies (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R) and limb malformation (L)) and MURCS (Müllerian (Mu), renal (R) and cervical somites dysplasia (CS)) in which these malformations are distinctive features. Etiologies for both VACTERL and MURCS are still mainly unknown (6,7), while reports of individuals with features of both conditions suggest the possibility of shared mechanisms for pathogenicity (8–10). CAKUT (Congenital anomalies of kidney and urinary tract), uterine malformation and costovertebral anomalies/congenital scoliosis, in their isolated presentation and as part of VACTERL or MURCS association, are mainly sporadic. However, familial occurrence is reported in 9-15% of individuals arguing for predisposing genetic factors (4,7,11–13). Variable expressivity and incomplete penetrance are observed, as illustrated in families with VACTERL or in families with renal abnormalities and/or MRKH (Mayer-Rokitansky-Küster-Hauser) syndrome (14,15), which poses challenges to the identification of new genes associated to these disorders. In isolated, or mildly syndromic CAKUT, next generation sequencing analyses have revealed high genetic heterogeneity with approximately 60 causal genes identified so far, that explain 11-20% of cases (16,17). In comparison, only a few genes have been involved in spondylocostal, renal and uterine malformations, including *TBX6* (deletion 16p11.2,

spondylocostal dysostosis type 5 (18,19)), *GREB1L* (Renal hypodysplasia type 3 #617805) (20), *MNX1* (Currarino syndrome (#176470)), *SALL1, DACT1 (*Townes-Brocks syndrome 1 and 2 (#107480;#617466)) (17) and *CDX2* (21). Unsolved familial cases suggest that new genes still have to be uncovered. By whole exome sequencing in families with recurrence of uterine and kidney anomalies, we identified *NR6A1* as a new candidate to explain predisposition to some of these various malformations.

NR6A1 (Nuclear receptor subfamily 6 group A member 1) is an orphan nuclear receptor highly conserved in vertebrates. The protein is essential for embryonic development, knockout mice being lethal at day 10.5 with severe defects in somitogenesis, abnormal chorioallantoic development, abnormal development of the ventral structures and defects in neural tube closure (22). Conditional deletion of *NR6A1* within axial progenitors in mice induces a decrease in the number of thoracolumbar vertebrae in a dose dependent manner, and modifies the timely progression of posterior hox genes expression (23). Expression data in mice and xenopus confirms its role early in development, with transient, high, widespread expression during the mid to late gastrulation (E7.5 in mice) and early organogenesis stages. Consistent with the involvement of *NR6A1* in developmental processes of the trunk, but not the tail, expression is absent in the most posterior area (posterior to the posterior growth zone in mice, near the tailbud in xenopus) and high levels were detected in the anterior and trunk regions, in the neuroectoderm and underlying mesoderm (23–26). In zebrafish, the gene is duplicated (*nr6a1a* and *nr6a1b*), and comparison of expression patterns are indicative of a conserved function for *nr6a1a* and possible neofunctionalization for *nr6a1b* (27). NR6A1 acts as a constitutive transcriptional repressor and was shown to inhibit pluripotency genes (e.g. *OCT4, NANOG, CRIPTO-1)* during retinoic acid mediated differentiation of embryonic stem cells (28–31). *In vitro*, NR6A1 binds as homodimers, or oligomers, with high affinity to a direct

repeat of the DNA sequence AG(G/T)TCA (DR0) (32,33), and with lower affinity to the extended half-site TCAAG(G/T)TCA (33). The potential ligands of NR6A1 are currently unknown, hence its designation as orphan nuclear receptor.

NR6A1 has not been associated with any human disease so far. Here, we report deleterious heterozygous variants in *NR6A1* in multiple individuals with renal, uterine, and vertebral anomalies. By inactivating the zebrafish *NR6A1* orthologs, we show that *nr6a1a/b* zebrafish mutants display kidney and axial skeletal anomalies reminiscent of those found in humans.

RESULTS

Identification of *NR6A1* variants in individuals with uterine, kidney and costovertebral anomalies.

Exome sequencing analysis was conducted on three affected relatives with kidney malformations with/without congenital uterine and costovertebral anomalies to investigate the hypothesis of a shared genetic basis for their congenital anomalies (Figure 1a-Family 1). The proband was a 46,XX female diagnosed with Mayer-Rokitansky-Küster-Hauser syndrome type 2. She had a single pelvic kidney requiring surgery at 9 months of age for a vesicoureteral reflux. The ovaries were both present in an ectopic position, located at the origin of the psoas muscles. Endocrinological workup was normal at age 13 with no hyperandrogenism. Development of the secondary sexual characteristics was normal. Spine and chest x-rays revealed the presence of 11 thoracic vertebrae instead of 12, with only 10 ribs on the right, and severe hypoplasia of the eleventh rib on the left (Figure 1b). There were no dysmorphic features and the patient was developmentally or cognitively normal. Microarray (180k SNP array) was negative. The family history is significant for a sister who was diagnosed with right renal hypoplasia and left

compensatory renal hypertrophy. She had no uterine malformation. In addition, the mother had a hemi-uterus with a single median pelvic kidney and one single right ovary. She underwent kidney transplantation at age 54 due to end-stage renal insufficiency. On imagery, the native kidney was atrophic with loss of differentiation. Exome sequencing was performed on the three affected family members; variants were filtered for rare (MAF<0.01) heterozygous variants shared by all three relatives. Variants in genes were prioritized by gene function (those known to be associated with CAKUT/uterine/vertebral anomalies in mammals), dominant inheritance, constraint metrics and *in silico* prediction. Based on this analysis, a missense variant in *NR6A1*, c.1175T>G; p.(Met392Arg) was selected as the most compelling candidate. First, *NR6A1* has been associated with variation of thoracolumbar vertebral number in mammals (23,34). Second, it is highly constrained for loss-of-function variation in gnomAD, with observed/expected value (LOEUF:0.19) (35), indicating such variants are not tolerated in the population. Its high pHaplo (0.99) and pTriplo (0.94) scores, suggest that the gene is likely to be sensitive to decreased DNA dosage (i.e., haploinsufficiency) and increased DNA dosage (i.e., triplosensitivity) (36). Finally, c.1175T>G is not observed in gnomAD v4.1 and is predicted to be deleterious by several *in silico* tools (CADD score: 29.6 ; REVEL score: 0.97 (deleterious strong); AlphaMissense:0.996 (deleterious strong)). The variant is located in the ligand binding domain (LBD) of NR6A1 and the substitution replaces a highly conserved methionine that is hydrophobic and uncharged by a hydrophilic and charged residue, an arginine. The Grantham's distance between both aminoacids is 91 [0-215]). The hydrophobic residue, conserved amongst nuclear receptors, contributes to the folding of the protein by forming van der Waals contacts between the 8th and 10th α-helices (37,38). Hence, changes of physicochemical properties of this amino acid likely alters the tertiary structure of the protein which may impact the affinity of the DNA binding, the homodimerization or interaction with co-repressors. Given the *in silico* data, the crucial

role of *NR6A1* in embryonic development, alongside phenotypic resemblances in the proband to the vertebral anomalies observed in conditional knockout *Nr6a1* mice, we hypothesized that the heterozygous missense variant found in this family may underly their malformations.

Submission to Genematcher (39) allowed to identify the second case, a male newborn who died very shortly after birth due to respiratory insufficiency in the context of bilateral renal agenesis, anhydramnios and intrauterine growth retardation, which were identified at the second trimester ultrasound. Family history was unremarkable. A heterozygous *de novo* variant, c.439C>T; p.(Gln147*), was identified in *NR6A1* by trio exome sequencing. X-rays and autopsy were not performed (Figure 1a. Family 2). In addition, another heterozygous *de novo* variant, c.800dup;p.(Thr268Hisfs*6) in *NR6A1* was previously reported by Boissel et al. (40) in a male fetus with bilateral renal agenesis, absence of the ureters, a unique umbilical artery and a retroesophageal right subclavian artery. Both variants generate premature termination codons, and hence, are likely to induce nonsense-mediated mRNA decay. Structural analysis suggests that any residual mRNA would encode a protein missing the entirety or most of the LBD domain, resulting in loss-of-function of the protein (41) (Figure 1a).

To identify additional individuals with *NR6A1*-associated disease, we performed targeted sequencing of *NR6A1* in a cohort of 68 individuals with MRKH and identified a heterozygous variant, c.196C>T; p.(Arg66Cys), in a 46,XX female with a left ectopic kidney, and a history of vesicoureteral reflux requiring surgery (Figure 1a- Family 3). Spine X-ray was normal. Parental samples were unavailable for segregation studies. The c.196C>T; p.(Arg66Cys) variant is rare in the population database gnomAD (11 heterozygous males/801566 and 3 heterozygous females/812472) and is predicted

deleterious *in silico* (CADD score:33; REVEL score: 0.92 (Moderate); AlphaMissense: 0.992 (Strong)). The variant is located in the DNA binding domain, in the first zinc finger subdomain, and affects a highly conserved arginine, that is substituted by a cysteine (Grantham's distance=180 [0-215]), possibly altering the interaction with the DNA.

Decreased DNA binding affinity of Arg66Cys and Met392Arg NR6A1 proteins *in vitro*

In order to ascertain whether the two missense variants we identified could alter the binding of NR6A1 to its target DNA and therefore prevents its transcriptional activity, we performed an electrophoretic mobility shift assay (EMSA). The native or the mutated form of the hsNR6A1 protein fused to a Myc-tag polypeptide were *in-vitro* translated and their concentration were assessed by western blot (Supplemental figure 1). As probe we used a radiolabeled oligonucleotide consisting of a direct repeat with zero spacing (DR0) of the consensus sequence AGGTCA. The probe was incubated with either the native hsNR6A1 or Arg66Cys and Met392Arg hsNR6A1 proteins (Figure 1c). Whereas a strong complex was formed on the DR0 probe with the native NR6A1 (lane 2), the complexes formed with the mutated NR6A1 proteins Arg66Cys (lane 3) and Met392Arg (lane 4) were almost undetectable, only a weaker signal was visible upon extended exposure. The DNA binding specificity was verified by a competition assay (Figure 1d); indeed the addition of a 125 fold excess of unlabeled DR0 blocked formation of the protein-probe complex (lane 3) while the addition of an unlabeled Oct2A-binding oligonucleotide had no effect (lane 4). The presence of NR6A1 protein in the complex was further confirmed with the observation of a supershift in presence of anti–Myc antibody (lane 5). Together these results showed that while native NR6A1 binds specifically DR0 sequence with high affinity, the mutations Arg66Cys and Met392Arg strongly decrease this affinity. It therefore suggests that the identified missense variants can prevent NR6A1 activity.

Loss of Nr6a1a/b in zebrafish cause skeleton anomalies and abnormal kidney morphology.

To evaluate the impact of NR6A1 loss-of-function, in particular in urogenital tract and skeleton formation, we inactivated the zebrafish orthologs *nr6a1a* and *nr6a1b* by CRISPR-Cas9. The higher degree of sequence homology between *nr6a1a* and *NR6A1* and the similarities in the pattern of *nr6a1a* expression compared to other species suggested that most of the conserved functions would be controlled by *nr6a1a*. However, the high similarity in the DNA binding domain between Nr6a1a and Nr6a1b did not exclude overlapping function in gene regulation. Moreover, although expression of *nr6a1b* occurs later, after 10 hours post fertilization (hpf), and is more restricted (Supplemental figure 2), both genes are expressed in same regions of the trunk (neural tube, paraxial and lateral mesoderm) during somitogenesis. In order to detect compensatory effect of the duplicated genes, phenotypes of single and double null mutants for *nr6a1a* and *nr6a1b* were analyzed.

No alteration was noted in *nr6a1b* $^{ulg085-/-}$ embryos and these mutants reached adulthood without any obvious defects. However, only a low percentage (around 4-10%) of *nr6a1a*$^{-/-}$ survived until adulthood. *Nr6a1a* $^{ulg083-/-}$ homozygous embryos displayed transient pericardial oedema from 30 to 56 hpf. The homozygous mutants that reached adulthood displayed visible morphological anomalies such as shorter trunk and absence of the anal fin (Figure 2a). Unilateral or bilateral aplasia or hypoplasia of the pectoral fin were also observed in a low percentage (6%) of *nr6a1a*$^{-/-}$ larvae. Homozygous mutants were slightly shorter compared to siblings (9.2 % shorter in average), with a disproportionate reduction of the anterior region of the trunk (10% reduction in average relative to the size) (Figure 2a). The homozygous females were infertile. Double *nr6a1a* ^{ulg083}*/b* ^{ulg085} homozygotes died before 16 days post fertilization (dpf). They presented the same morphological anomalies than the *nr6a1a*$^{-/-}$ mutants with an increasing penetrance of the

pectoral fin phenotype supporting a compensatory role for *nr6a1b* in some tissues (Supplemental figure 3).

To characterize the skeletal defects, MicroCT scans were then performed on adult *nr6a1a*$^{-/-}$ mutants and wildtype females. *Nr6a1a*$^{-/-}$ mutants presented a reduced total number of vertebrae from 31 in wildtypes to 26 (+/-1) in mutants (n=4). Interestingly, the five missing vertebrae are located in the trunk and correspond to precaudal vertebrae from which the ribs originate from. This is consistent with the shortened trunk region we observed (Figure 2b and Supplemental figure 4). The dorsal fin radials start at the level of the penultimate precaudal vertebrae, corresponding to the 4th and 9th non-Weberian precaudal vertebra in mutants and wildtypes, respectively. The axial skeleton of some mutants presents other defects, such as abnormal, irregular shape of the ribs and unfused hemal arches of some caudal vertebrae (Supplemental figure 4). Moreover, we confirmed the absence of the anal fin rays (Figure 2b).

Because of anomalies of the female genital tract in humans with *NR6A1* variants and infertility in *nr6a1a*$^{-/-}$ zebrafish females, we questioned the possibility of gonadal duct anomalies in surviving adults. Formation of the gonadal duct starts around 25dpf in teleost, with firstly the formation of the upper part of the oviduct in continuity with the ovarian cavity, progressive elongation and secondarily, junction to a lower part resulting from invagination and cavitation of the urinogenital papillae around 50dpf (42,43). Incubation of whole fish in a lugol solution allow for visualization of soft tissue by MicroCT scan. Using this contrast agent, we were able to visualize the oviducts. The oviduct seemed formed in the *nr6a1a*$^{-/-}$ females, however the channel looked obstructed compared to controls (Supplemental figure 5). Unfortunately, the precocious lethality of the double

mutant did not allow to address the impact of a total loss-of-function of *nr6a1* alleles on the gonadal tract.

As congenital anomalies of the kidneys were present in humans (aplasia/hypoplasia/ectopia), we investigated the kidney structure in the adult *nr6a1a*$^{-/-}$ mutants. To easily visualize the renal tissue, we injected intraperitoneally a solution of Rhodamine-dextran in 4 wildtypes and 4 *nr6a1a*$^{-/-}$ mutants. This small molecule is uptaken by the kidney proximal tubules within a day post injection. It colors the organ in red, and allows a functional assessment of the proximal tubules (44). The adult zebrafish kidney is a flat organ located on the dorsal body wall. When viewed from a ventral perspective, the wildtype kidney has a distinctive curved morphology, consisting of head, trunk and tail regions. This typical shape is perturbed in the *nr6a1a*$^{-/-}$ mutants, the trunk segment being larger. To determine if there was a change in the size of the kidney relative to the fish size, we measured the overall surface of the kidney and made a ratio on the length of the fish. Hence, we showed a decrease by 24% of the kidney size in the *nr6a1a*$^{-/-}$ mutants compared to wildtypes (p-value=0.017) (Figure 2c). The proximal tubule of the *nr6a1a*$^{-/-}$ mutants seemed functional as we could observe uptake of Rhodamine-dextran as in wildtypes (Supplemental figure 6).

Together, our data show that total inactivation of NR6A1 orthologs is embryonically lethal and that inactivation of Nr6a1a alone is sufficient to induce costovertebral defects and kidney hypoplasia. Those anomalies being similar to those observed in human individuals with *NR6A1* variants, it strongly suggests that these variants are responsible for their syndrome.

Nr6a1a/b are required for proper pronephros development.

Given the developmental origin of the malformations in individuals with *NR6A1* variants and as we showed that *NR6A1* is essential for embryonic development, we further investigated the function of the gene in kidney development. The zebrafish pronephros, as the earliest nephric stage, contains two nephrons sharing numerous genetic, structural, and functional aspects with the mammalian nephrons. From 24hpf, it consists of glomeruli fused at the embryo midline and of tubular epithelium extending from the glomerulus to the cloaca where the gastrointestinal tract also opens up (Figure 3a-b). Interestingly, the terminal end of the pronephros (named pronephric duct, PD) was abnormal in *nr6a1* mutants with variable expressivity as shown by brightlight microcoscopy at 5 dpf (Figure 3a). The double homozygous mutants were the most severely affected, with some larvae showing a blind end of the digestive tract and an indistinguishable pronephric duct orifice. A milder phenotype was observed in the simple *nr6a1a*$^{-/-}$ mutants, with presence of the gut opening but abnormal cloaca with a not well-defined end of the pronephric duct.

From 24hpf onwards, the tubular epithelium of the zebrafish pronephros is subdivided into segments that are functionally homologous to segments of the mammalian nephron: two proximal tubule segments (proximal convoluted tubule (PCT) and proximal straight tubule (PST)), connected to the glomerule (G) by the neck segment (N) and two distal tubule segments (distal early (DE) and distal late (DL)) connected to the cloaca by a short segment (the pronephric duct (PD)) (Figure 3b) (45). Double whole-mount in situ hybridization (WISH) with segment-specific marker gene and the somite boundary marker *xirp2a* (45) were performed at 24hpf and/or 48hpf on embryo from single and double heterozygous mutants incross. *Clcnk* and *gata3* are normally expressed in the distal segments (*clcnk*: DE-DL-PD; *gata3:* distal part of the DL-PD and corpuscule of Stannius (CS)). The expression of both markers is extended anteriorly by around 2 somites width in

nr6a1a$^{-/-}$ (Figure 3 and Supplemental figure 7). Hence, *clnck* expression that usually starts at the level of the 9th somites is seen as anterior as the 7 somites in the mutant (average extension of 1.3 somites width (CI 95%:0.62-1.98). *Gata3* is extended anteriorly by 2.3 somites width in average (CI 95%:1.57-3.06). Conversely, expression of *trpm7*, which labels the proximal straight segment, is reduced. *Trpm7* is expressed from the sixth somites, it extends to the 9th somite in wildtype but stops between the 7th and the 8th somites in homozygous mutants (average length decreased by 1.4 somites width, CI95%:1.08-1.71). Finally, an extended expression was observed in mutants (selected as showing no *tbx5* expression) compared to wildtypes for *evx1*, a gene normally expressed at 24hpf in the posterior gut, the cloaca and the lateral mesoderm surrounding the end of the pronephric duct (Supplemental figure 7) (46). No differences were observed in the expression of markers labeling the glomerules (*wt1a*, *nephrin*) or PCT (*slc20a1*) in *nr6a1a*$^{-/-}$ mutants compared to wildtype. Double homozygous (*nr6a1a*$^{-/-}$; *nr6a1b*$^{-/-}$) have similar expression of *gata3* to the simple *nr6a1a*$^{-/-}$ mutant in standard conditions (Supplemental figure 7). These results show the involvement of *nr6a1a* in nephron patterning with a substantial effect on the intermediate and distal segments.

Nr6a1a/b loss-of-function modify posterior Hox genes expression in zebrafish

The phenotype observed in the patterning of the pronephros as well as the missing vertebrae are reminiscent of antero-posterior patterning defects. As in vertebrates Hox genes provide the major positional information along the anteroposterior axis, we investigated hox genes patterning in *nr6a1a/b* mutants. We focused on *Hoxa9–13* and *Hoxd9–13* as these posterior Hox determines notably the patterning of the female genital tract (47,48), as well as vertebrae and ribs patterning (49), metanephros formation and nephron segment identity (50,51) in mammals. Posterior hox paralogs demonstrate collinear expression in the trunk along the anteroposterior axis of the zebrafish embryo;

they are expressed in ventral and lateral mesoderm with anterior boundaries depending on each particular hox gene (Figure 4 and Supplemental figure 8) (52,53). WISH were first performed for *hoxa9b* in 31 hpf embryo from *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ incross. Compared to wildtypes, the domain of expression of *hoxa9b* was extended anteriorly when *nr6a1a* was inactivated (*nr6a1a*$^{-/-}$*).* The loss of one or two *nr6a1b* allele did not exacerbate the phenotype*,* showing that *nr6a1a* is the main contributor to the phenotype at least for *hoxa9b* (Figure 4a). Similar results were shown for *hoxa10b* at 18hpf (18 somites stage), with no differences following *nr6a1b* inactivation alone (Supplemental figure 8). As *nr6a1b* could still compensates *nr6a1a* for other targets, we systematically investigate the expression of other posterior hox gene in a *nr6a1b*$^{-/-}$ genetic background. WISH were performed at 18hpf and 24 hpf for *hoxa10b (n=18), hoxa11b (n=17), hoxa13b (n=17) or hoxd11a (n=17).* Similar to *hoxa9b*, an anterior shift of expression was observed for those markers. Embryos were genotyped based on their phenotype and we showed that 100% of the embryo with a pattern of expression shifted anteriorly were homozygous for *nr6a1a* and that all the *nr6a1a -/-* embryo exhibit this shift (Figure 4b and Supplemental figure 8). Interestingly, *hoxb13a* whose expression is restricted to the posterior extremity of tail in wildtype embryos, is ectopically expressed in the double *nr6a1a;nr6a1b* mutants at 24hpf (Figure 4c). Indeed, ectopic expression appears just above the yolk extension in the posterior endoderm and/or mesoderm with the inactivation of both *nr6a1a* and *nr6a1b*. Very mild ectopic expression was detected in *nr6a1a* single mutants (Figure 4c). Finally, we tested several paralogues of the anterior and central hox clusters *(hoxb1a, hoxa2b, hoxb3a, hoxd3a, hoxb4a* at 21 hpf*; hoxb6b, hoxb8b* at 31hpf) and we did not detect any differences between mutants and wildtypes. These findings indicate that *nr6a1a* globally regulates the expression pattern of several posterior *hox* genes from paralogous groups a,b and d in zebrafish, resulting in ectopic expressions that extend anteriorly in mutants.

DISCUSSION

In this study, we report for the first time an association between the *NR6A1* gene and congenital anomalies in humans. Our findings shed light on the role of *NR6A1* in human development, heterozygous deleterious variants in the gene predisposing to renal agenesis/hypoplasia, spondylocostal and uterine malformations. The alteration in kidney and skeleton observed in our knockout *nr6a1a/b* zebrafish model support the gene's causality. Functional assessment of the two *NR6A1* missense variants identified in human subjects revealed decreased binding to the consensus DNA sequence, confirming a partial loss-of-function for the mutated proteins. Meanwhile, the two protein-truncating variants, found in fetuses, likely result in mRNA decay and *NR6A1* haploinsufficiency, or alternatively would produce a truncated protein lacking the LBD domain. Individuals harboring heterozygous protein-truncating variants exhibit severe defects like bilateral renal agenesis, while missense variants are linked to non-lethal malformations such as unilateral renal agenesis, renal hypoplasia, and kidney ectopia with or without uterine and skeletal anomalies. In our zebrafish model, we observed a dose-sensitive effect, where some phenotypes become progressively more severe or penetrant in *nr6a1a-/-* with the loss of additional *nr6a1b* alleles; this is the case for the malformations at the level of cloaca and of the pectoral fins. A dosage effect was also demonstrated previously during somitogenesis in mice (23), and is supported by the high pHaplo (0.99) and pTriplo (0.94) scores of *NR6A1*. Further reports on individuals with *NR6A1* variants are warranted to elucidate genotype-phenotype correlations. Nonetheless, our results suggest that the variable expressivity of the phenotype possibly depend on NR6A1 dosage, influenced in part by the type of variant.

Amongst the clinical features, the uterine malformations were presents in three of four female individuals (MRKH syndrome in two, and hemi-uterus in one). These anomalies are suggestive of a defect in the formation or elongation process of the Müllerian ducts. Similarities in development between the oviduct in zebrafish and genital tract in mammals have been suggested. For instance, the zebrafish orthologue of *WNT4*, a well-known causal gene of MRKH syndrome, present a conserved role in gonadal ducts formation (42,54). Although no obvious anomalies were observed in the gonadal duct of the 3-month-old *nr6a1a*$^{-/-}$ female zebrafish, we cannot rule out reduced penetrance of the phenotype due to the low number of surviving single *nr6a1a* homozygous. Moreover, loss of both orthologues may be required for the phenotype, that cannot be ascertained given lethality of double homozygous embryos before the onset of gonadal duct formation. However, the cloacal anomalies and defects of the terminal part of the pronephros and hindgut suggest that elongation of the oviduct might be affected in the absence of Nr6a1 protein.

A decrease in the number of ribs and ribs bearing vertebrae was observed in our zebrafish mutants, reminiscent of the defects observed in conditional knockout mice (23). Additionally, we observed a similar phenotype in one human subject, with fewer thoracic vertebrae and ribs. While 3-6% of the population may have 11 pair of ribs and vertebrae instead of 12 (55–57), the presence of 10 ribs is uncommon in humans. Interestingly, this reduction in the number of thoracic vertebrae and ribs might be a phenotypic clue to suspect the presence of *NR6A1* variants in humans with renal agenesis and uterine malformation.

Besides regulation of the number and segmentation of thoracolumbar vertebrae, our experiments in zebrafish supports that NR6A1 is also essential for the formation of other

organs in the region of the trunk, with additional roles in kidney development and proper fusion of the pronephros and hindgut to the cloaca. The anterior shift observed in *hoxa9-13* expression may link our observations in zebrafish to the malformations observed in humans. During the formation of thoraco-lumbar somites, *Hoxa10* expression in presomitic mesoderm determines the transition between ribs-bearing and lumbar vertebrae in mice (58) . A more rostral *HOXA10* expression, may result in a decreased number of ribs and ribs-bearing vertebrae. Moreover, precocious expression of *Hox13* paralogous in mice was shown to abruptly terminate patterning and elongation resulting in posterior truncation, and cloacal anomalies (anal atresia, anorectal agenesis or abnormal communication/septation between the bladder and the hindgut) (59,60) and their ectopic expression causes kidney agenesis (61) and posterior homeotic transformation of the female genital tract (62). Significantly, our experiments show ectopic expression of *hoxb13a* in the posterior mesoderm and endoderm in zebrafish mutants, and this may affect elongation processes in the urogenital region. Finally, in addition to posterior Hox genes, NR6A1 represses directly or indirectly other signaling molecules or transcription factors (63), which may also impact the development of the kidneys and uterus. Further investigation will be needed to identify the downstream targets of NR6A1 that explain the pronephros patterning defect.

In conclusion, we report *NR6A1* as a new candidate gene for renal agenesis, uterine malformation and vertebral anomalies in human. Identification of additional individuals will be necessary to confirm causality and delineate the spectrum and penetrance of anomalies associated with *NR6A1* heterozygous variants. Interestingly, the observation of cloacal malformation and pectoral fin anomalies in our zebrafish model, in addition to renal and vertebral malformations, makes *NR6A1* a compelling candidate gene for VACTERL association, in which anorectal malformation and radial ray defects are typical

features. In individuals with MRKH syndrome who fulfill the criteria for an additional diagnosis of VACTERL, anorectal and renal malformations were always present, and vertebral anomalies reported in the majority of cases, highlighting the possibility of shared genetic factors and developmental pathways to explain the co-occurrence of these malformations (10). Compared to mice, zebrafish null mutants for *nr6a1a/b* exhibit later lethality in their development, allowing the investigation of late organogenesis processes. Furthermore, the survival of single *nr6a1a* homozygous mutants until the adult stage enables the observation of milder or late-onset phenotypes. In contrast, mouse models (likely through conditional knockout) will be valuable for studying the role of NR6A1 in ureteric bud branching and metanephros morphogenesis, as well as Müllerian ducts formation and patterning. Therefore, both models will complement each other in delineating further the functions of *NR6A1* in development.

METHODS

Clinical proband samples and sequencing

Blood or salivary samples and pedigree information were collected after informed consent from individuals or their guardians. Whole exome sequencing was performed on blood, saliva or fetal tissue samples derived-DNA using the Ion AmpliSeq Exome target enrichment kit on an Ion Proton System sequencer for Family 1 and the NovaSeq 6000 Illumina sequencing platform for Family 2, generating 150bp reads (paired-end with Illumina platform) that were mapped to the human reference genome (NCBI build 37/hg19). The average exome coverage was above ×50. Variants were annotated and filtered using Alissa Interpret v2.1 (Agilent Technologies) (Family 1). Variants were filtered based on a minor allele frequency <0.5% in the population database gnomAD, their presence in exonic regions ±20bp of exon boundaries and a predicted change at the protein level (synonymous variants excluded). Variant in genes were prioritized by gene function (those known to be associated with CAKUT/uterine/vertebral anomalies in mammals), suspected mode of inheritance (dominant in Family 1, dominant or recessive in Family 2) and constraint metrics. In addition, *in silico* prediction tools such as PolyPhen-2, SIFT, PROVEAN, Mutation Assessor and Mutation Taster were used to assess the potential functional impact of missense variants; those predicted to be deleterious by 3 to 5 *in silico* prediction tools were selected. For each variant of interest, we further queried the CADD, REVEL and AlphaMissense scores (64–66). For targeted sequencing, single-molecule molecular inversion probes (smMIPs; EasySeqNGS- customized Targeted Capture Kit, Nimagen) were designed to target the exons ±20 nucleotides of intronic/exonic boundaries of *NR6A1* (NM_033334.4). Except for exon 1, the entire coding sequence was covered more than x100. Single nucleotide variations were interrogated using the Seqnext (SeqPilot) software. The variants identified were confirmed by Sanger sequencing. Variants in the

NR6A1 gene are described for reference sequence NM_033334.4, which encodes for the NR6A1 reference protein NP_201591.2 using HGVS nomenclature (www.hgvs.org)

Electrophoretic mobility shift assay.

The electrophoretic mobility shift assays (EMSA) were performed essentially as described by Greschik et al. (38) using a DR0 double stranded oligonucleotide (AGCTTCAGGTCAAGGTCAGAG) end-labbeled with 32P-dATP and Klenow enzyme. The human NR6A1 protein, as well as the R66C and M392R mutated proteins were synthesized *in vitro* using reticulocyte lysates (Promega). To that end, the coding sequences of the human wildtype NR6A1 cDNA and of the missense R66C and M392R mutants (obtained from VectorBuilder) were inserted in the expression vector pCS2-MT just downstream the SP6 promoter and fused to the MYC-tag at the C-terminus of the NR6A1 proteins. SP6 polymerase was used to produce *in vitro* the corresponding mRNA (Invitrogen mMessage Machine kit) which were next translated *in vitro* using the reticulocyte lysate following manufacturer's instructions (Promega). Production of proteins was verified by western blot using a rabbit MYC antibody (16286-1-AP, Proteintech). For EMSA assay, proteins were incubated in buffer containing 30mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 10 % glycerol, 1µg of poly (dI-dC) with 0.1 ng of DR0 32P-labelled probe for 20 min at room temperature. For competition and supershift assays, unlabelled oligonucleotide or MYC antibody were preincubated for 10 min with the proteins in the same buffer before addition of the DR0 probe. The reactions were loaded on a 6 % non-denaturing polyacrylamide gel in 0.5X Tris-borate-EDTA running buffer at room temperature. After the run, gels were dried and autoradiographed.

Generation of Crispr-Cas9 *nr6a1a/b* zebrafish mutants

All studies performed in Zebrafish were approved by the ethical committee of Liège university (protocol 2404).

CRIPSR-Cas9 mediated genome editing was used following the IDT protocol 'Zebrafish embryo microinjection Ribonucleoprotein delivery using the AltR™ CRISPR-Cas9 System', contributed by Jeffrey Essner, PhD (67). CRISPR-Cas9 crRNA targeting exon 3 of *nr6a1a* (GRCz11Ensembl Transcript ID: ENSDART00000191653.1; exon 3, 5' - CAGGACTGCACTATGGCATT -3') and the crRNA targeting exon 3 of *nr6a1b* (GRCz11Ensembl Transcript ID: ENSDART00000011096.8 ; exon 3 5'- GCACTATGGTATTATTTCCT-3') were designed using ChopChop to generate F0 mosaic embryos. The Alt-R-crRNA, Alt-R CRISPR-Cas9 tracrRNA and Alt-R Cas9 Nuclease V3 were obtained from Integrated DNA technologies.

The null mutation nr6a1a$_{ulg083}$ is a 5bp deletion in exon 3 (ACCGCGCCACAGGACTGCACTATGG) leading to a frameshift in the coding sequence and generating a restriction enzymatic Btslv2 site. For genotyping, the 249pb fragment was amplified using the primers CAGGCTGAGCAGCGCTCTTGTC and TCCAGCGTGTGTGTTTCTCTCAC and terra Taq polymerase (Takara) followed by Btslv2 digestion. The null mutation nr6a1b$_{ulg085}$ is an indel (CCTGCACTATGGTATTATTTCCTG to CCTGCATGCTATATATTTCCTG) leading to a frameshift and disrupting a BsgI restriction site. Genotyping this variant was done by amplifying the 387 bp fragment using primers TGCCCAATGGTTGGTTTGGCTACA and GTGTGTCTCACCCTTGCGGTTCATG followed by BsgI digestion.

Dissection and measurement of the adult zebrafish kidney after labeling of the proximal tubule with fluorescent Rhodamine-dextran

15 months-old adult fish were anesthetized in tricaine and injected with 10 µl of Rhodamine-dextran 3.5% in the peritoneal cavity. Three days later, the kidney was

dissected as described previously (44). The adult kidney (mesonephros) was gently detached from the dorsal wall, placed on a glass slide, flattened and covered by a glass coverslip. Rhodamine-dextran compounds, incorporated by the epithelial cells in the proximal comvoluted tubule (PCT), allow: - 1) to better delineate the mesonephros colored in dark-red; - 2) to functionally assess the endocytic uptake by the proximal tubule cells. Pictures were taken with Leica fluorescent binocular (M165). Area measurements were performed with Fiji (ImageJ). For each fish, the ratio of kidney surface (µm2) to fish length (mm) was calculated and then normalized to that of wildtype fish. Graph and statistics (parametric t-test and p-value) were obtained with JMP-Pro 17.2.

MicroCT scan

Adult fish were anesthetized in tricaine and then incubated in 4 % paraformaldehyde for 2 days at 4°C. Fish were rinsed three times in PBS prior to being imaged for skeletal structures analysis. Imaging was done on a Bruker Micro-CT Skyscan 1172/G. The fish were fixed in home-made adapted cylindrical EPS containers and PBS was added to avoid dehydration over the duration of the scan. After mounting the sealed container on the scanner's sample stage, a relaxation time of 45 minutes was left prior to scanning. The voltage and intensity of the X-ray source were set at 100 kV and 100 µA respectively and a 0.5 mm Al filter was used. The exposure time was set at 300 ms and acquisitions proceeded over 360° with steps of 0.4°. The detector is a SHT 11Mp camera, set with 4x4 binning to obtain projections of 1000 by 666 pixels in size.

After this microCT scan, the same fish were stained by incubation in Lugol 2.5 % for one day at room temperature, washed three times in PBS for 5 minutes and scanned again for analysis of soft tissues. The same conditions were used for the setup and the scans except that the rotation step was reduced to 0.2° and the imaging limited to the abdominal zone.

After scanning, reconstruction was performed on the Bruker software NRecon. Further image analyses were performed upon processing the data with the visualization software Avizo with custom-made computing modules.

Whole-Mount Zebrafish in situ hybridization

Whole-mount is situ hybridization (WISH) of zebrafish embryos using digoxigenin- and FITC- labelled probes were performed as described previously (68,69). The expression patterns of *trpm7, gata3, clcnk, slc12a3, xirp2a* and *tbx5* were previously reported (45,70,71). The 3' part of cDNA of *hoxa9b* (GTGGTCCAGCAGCAGTCTCGTG and GGATCTAGCTTCGTCTCCGCAGG), *hoxd11a* (CGCAAGTCCAACCTGTTCGC and GGGCTCGAGTGCGACAAAGTC), *hoxb13a* (GTGAGCGTGCTATGACCACC and GGAGACTATCGTGTCGCGG) and *evx1* (AGCTTTGGGCACTTTGGCAGT and GCTGCACATTTCGGTGTGCTGC) were amplified by PCR using the corresponding primers and the cDNA were inserted downstream the T3 promoter in the reverse orientation for the production of antisense labelled RNA by *in vitro* transcription. WISH were performed on clutches of 40 or 80 embryos respectively obtained from incross of *nr6a1a*$^{+/-}$ fish or of *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ fish or of *nr6a1a*$^{+/-}$*nr6a1b*$^{-/-}$ fish. After WISH, about 20 stained embryos were photographed and genotyped as described above. Graph and statistics (student's t-test and p-value) were obtained with JMP-Pro 17.2.

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FIGURES

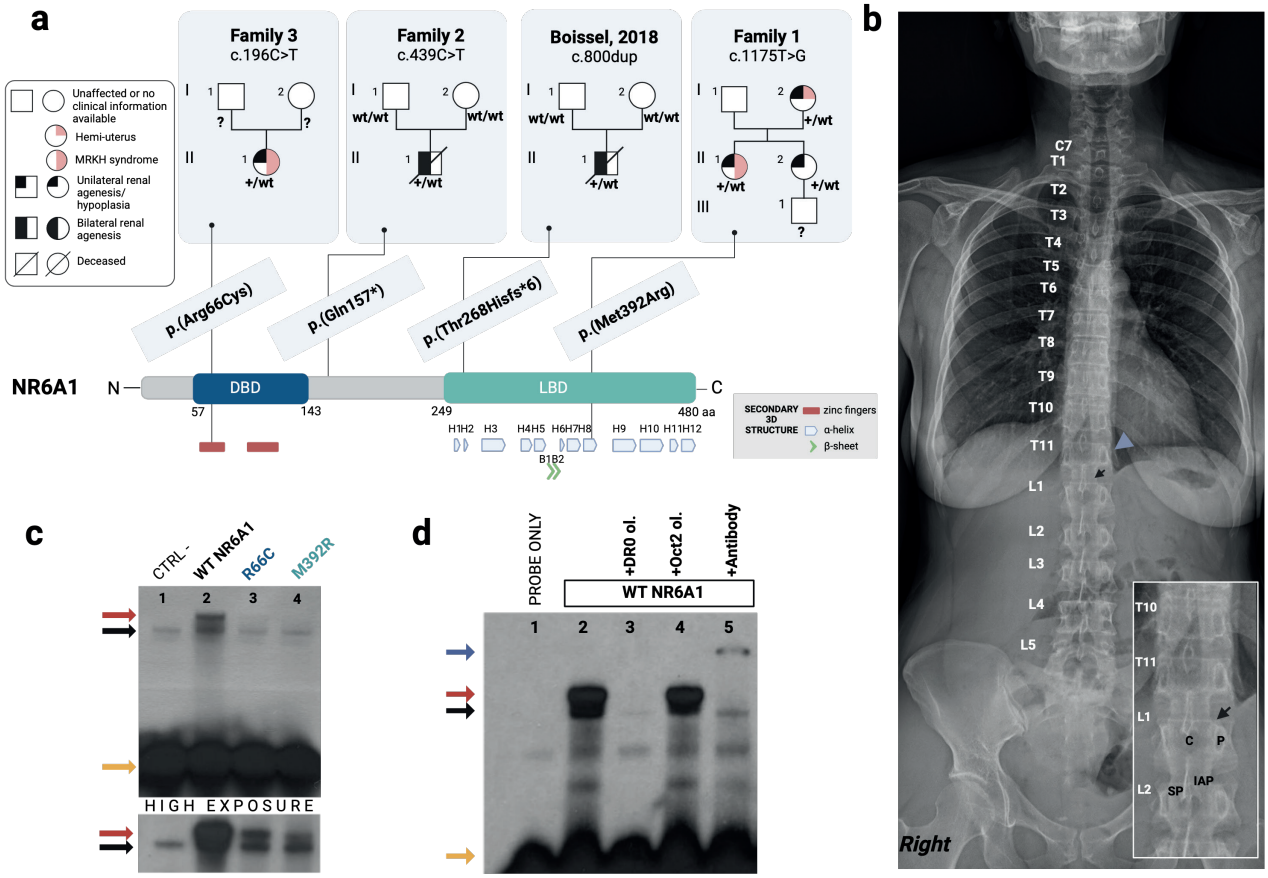

Figure 1. Identification of variants in *NR6A1* in four families with renal agenesis/hypoplasia with/without uterine and skeletal anomalies.

a. Pedigree and localization of the truncating and missense variants along the NR6A1 protein (NM_033334.4). The genotype of relatives is mentioned as "?" if not tested. NR6A1 is depicted with its main functional domains and 3D structure. The DNA-binding domain (DBD in blue) include two zinc fingers (red rectangles). The 3D structure of the ligand binding domain (LBD in turquoise) is composed of twelve alpha-helices (H1-H12) and two beta-turns (B1-B2).

b. X-ray of the spinal column (Family 1; individuals II:1) showing 11 thoracic vertebrae (T), 10 ribs on the right and a 11th hypoplasic rib on the left (blue arrow head), and a normal number of five lumbar vertebrae (L). The black arrows point to the interspace of the facet joint visible between the last thoracic vertebrae and the first lumbar vertebrae given its orientation.

c. Electrophoretic mobility shift assay with ^{32}P-radiolabeled DR0 consensus sequence, showing lower complex formation for the mutated hsNR6A1 proteins compared to controls (red arrow). An additional band (black arrow) is detected in the four samples corresponding to non-specific interaction of DR0 probe with proteins of reticulocyte lysate. The yellow arrow points to the unbound labeled DR0 oligonucleotides. The negative control (CTRL-) corresponds to incubation of probe with reticulocyte lysate without any mRNA. WT NR6A1, R66C and M392R correspond to wildtype and mutant NR6A1 proteins synthesized in reticulocyte lysates.

d. Competition analyses and supershift assay. The red arrow points to the band formed by the DR0-NR6A1 complex, the black arrow points to non-specific protein-DNA complexes from the reticulocyte lysate, the yellow arrow points to the unbound labeled DR0 oligonucleotides, and the blue arrow points to the shifted complex in presence of anti–Myc antibody.

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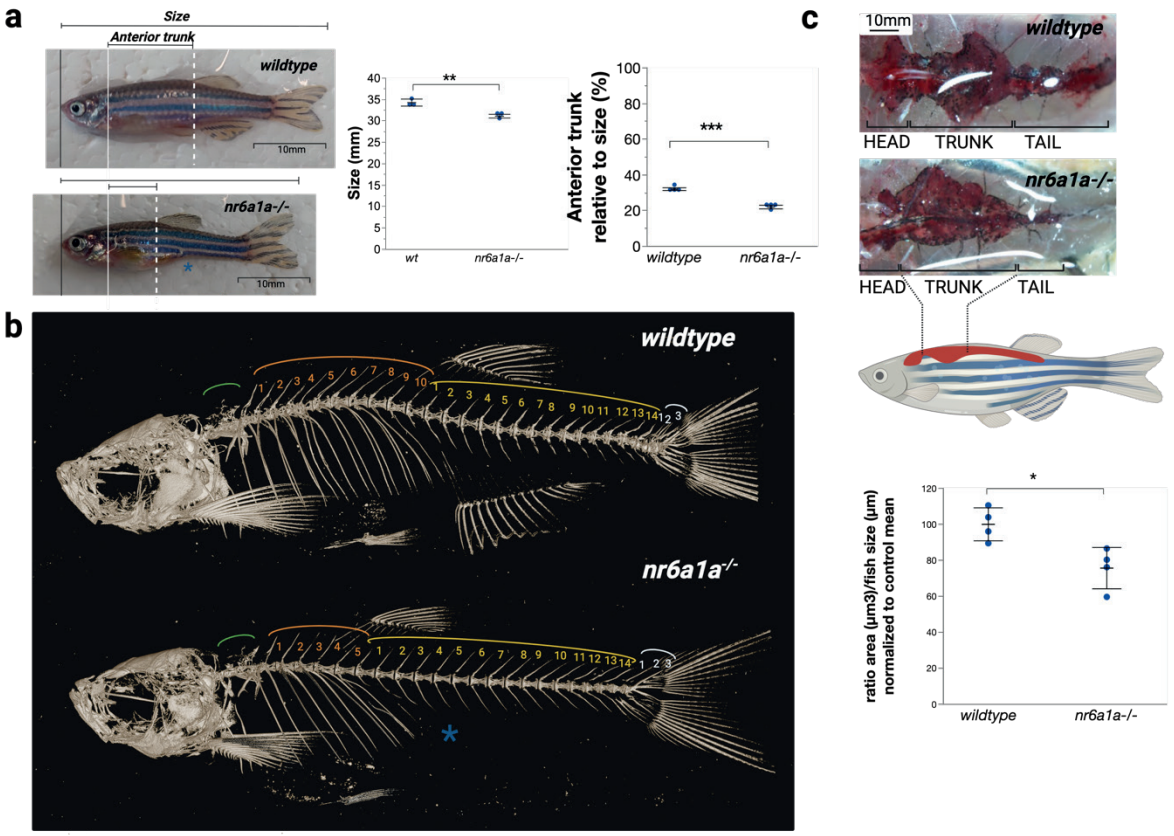

Figure 2. Smaller size, decreased number of ribs-bearing vertebrae and abnormal size and morphology of the kidney in *nr6a1a*$^{-/-}$ adult zebrafish.

a. Comparison of the size of wildtype (n=3) and mutant (n=3) siblings at 5 month ½ (mean size wildtype: 34.295mm; mean size mutants:31.125mm: difference: 3,17mm (CI95%:1.47-4.87) (p value=0.0095)). Comparison of the length of the anterior part of the trunk relative to the fish size between wildtypes (n=4) and mutants (n=4) (mean ratio wildtype 32.048 mm; mutants:22.036 mm; difference 10.01 mm (CI95%:8.43-11.59) (p value <0.0001).

b. X-ray microtomography of 3-month-old wildtype and mutants. The number of rib-bearing precaudal vertebrae (orange) is decreased in mutants (#5 instead of 10 in wildtypes). The rib-bearing precaudal vertebrae are recognizable by the presence of ribs, and the absence of hemal arches and spines, that are them specific of the caudal vertebrae (72). The number of caudal vertebrae (yellow, and light blue for the caudal fin vertebrae) is normal in mutants (#17). Absence of the anal fin (beginning at the 1st caudal vertebrae in wildtype) in mutants (*). The weberian vertebrae (vertebrae 1 to 4) are highlighted in green and seems normal although not investigated in details.

c. Mesonephros morphology of 15 month-old wildtypes and mutants. The mutants present an abnormal shape of the mesonephros, dissected after injection of

Rhodamine-dextran for visualization. Compared to wildtypes (n=4), the surface of the mesonephros relative to the fish size (after normalization by the control mean), is decreased by 24.35% (CI95%:6.15-42.54) (p value=0.017) in *nr6a1a*$^{-/-}$ mutants (n=4).
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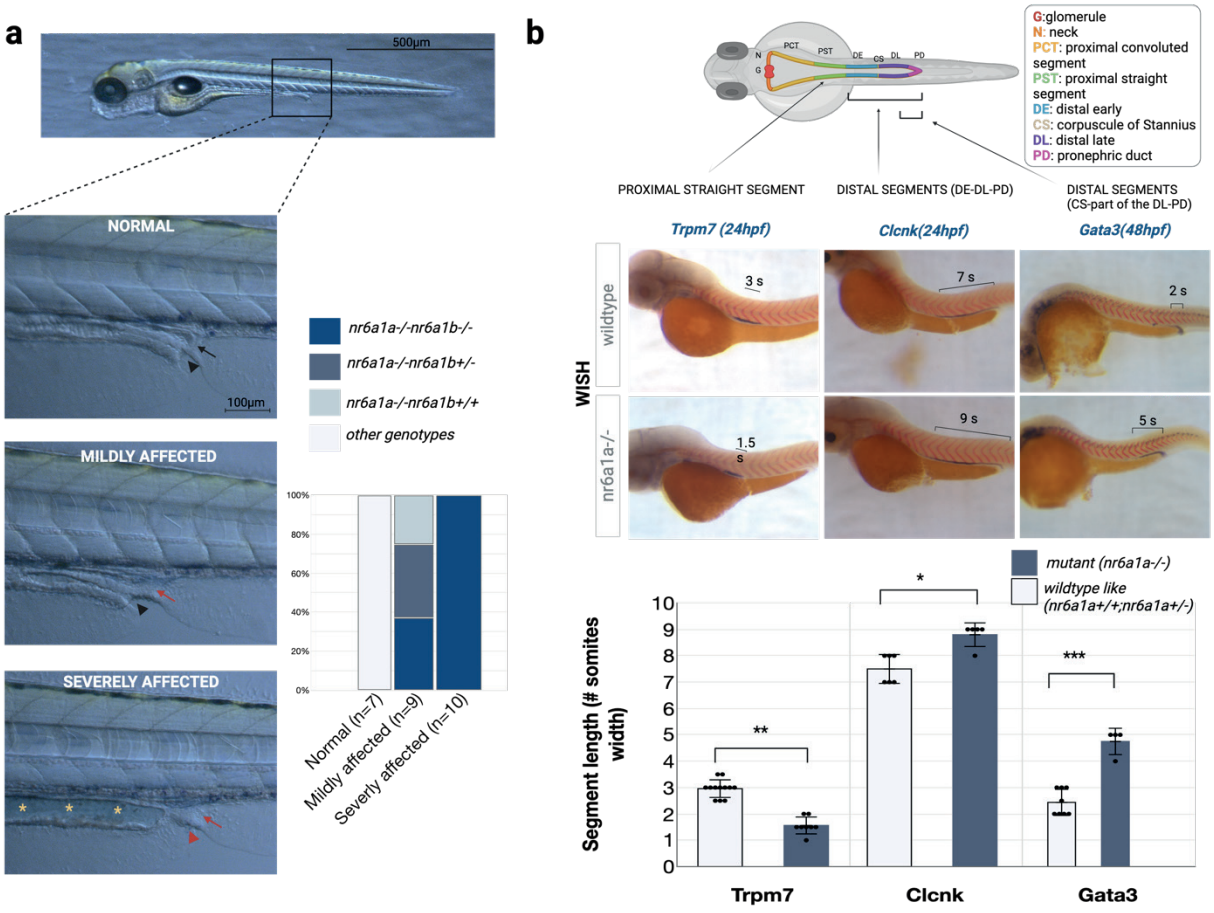

Figure 3. Defects in cloacal morphogenesis and pronephros patterning in *nr6a1a*$^{-/-}$ and *nr6a1a*$^{-/-}$ *nr6a1b*$^{-/-}$ mutants.

a. Brightfield images of the cloaca at 120hpf in embryos of *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ incross. Classification of the phenotype as normal if the cloaca presents a typical inverted U-shaped with a visible pronephric duct in its terminal part (black arrow), and a visible opening of the gut (black arrow head); mildly affected if the lumen of the gut reach the cloaca (black arrow head) whereas the terminal part of the pronephric duct is not visible (red arrow); severely affected if both ends are not visible (red arrow and red arrow head). Some severely affected embryos present dilation of the gastrointestinal tract (yellow stars), suggesting imperforation. 26 embryos were selected for genotyping (normal (n=7); mildly affected (n=9); severely affected (n=10)) Embryos showing normal morphology are wildtype or heterozygous for *nr6a1a* and show variable *nr6a1b* genotypes. Affected embryos are all *nr6a1a*$^{-/-}$ with severely affected embryo being homozygous for both *nr6a1a* and *nr6a1b*.

b. Double whole-mount in situ hybridization with the somite boundary marker *xirp2a* and the pronephros segment markers *trpm7, clcnk* and *gata3* in embryos from *nr6a1a*$^{+/-}$ incross. The width of one somite is used as reference to measure the length of the

expression domain. Expression in *nr6a1a*$^{-/-}$ mutants compared to wildtype/heterozygotes is increased with a difference of +2.3 somites ($p<0.0001$) for *gata3* (n=4 for *nr6a1a*$^{-/-}$ and n=8 for *nr6a1a*$^{+/- \text{ or } +/+}$),+1.3 somites ($p=0.0022$) for *clcnk* (n=5 for *nr6a1a*$^{-/-}$ and n=6 for *nr6a1a*$^{+/- \text{ or } +/+}$), and -1.4 somites ($p<0.0001$) for *trpm7* (n=8 for *nr6a1a*$^{-/-}$ and n=12 for *nr6a1a*$^{+/- \text{ or } +/+}$). Created with BioRender.com

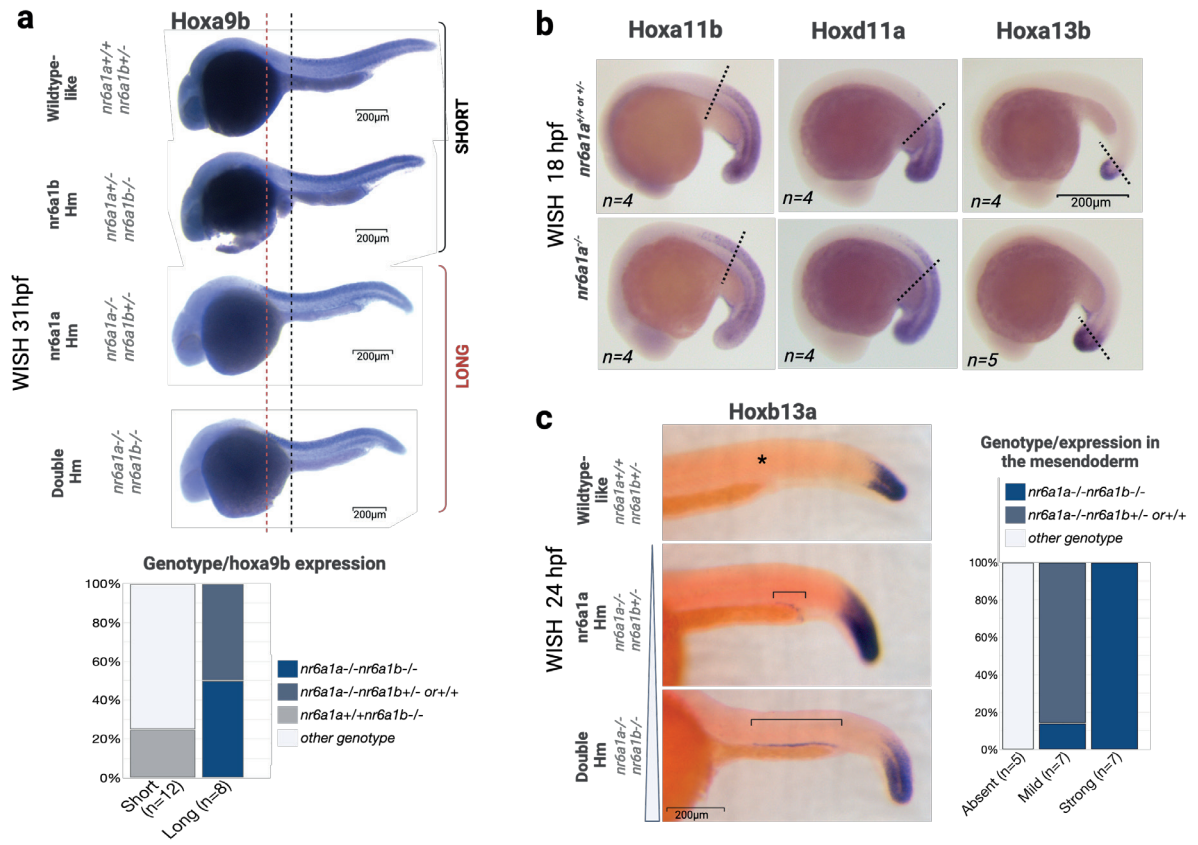

Figure 4. Abnormal patterning of posterior Hox in *nr6a1a*$^{-/-}$ and *nr6a1a*$^{-/-}$*nr6a1b*$^{-/-}$ mutants.

a. Whole-mount is situ hybridization with *hoxa9b* at 31hpf in embryos from an incross between *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ and *nr6a1a*$^{+/-}$*nr6a1b*$^{-/-}$ fish. A longer anterior axial staining (neural tube) was observed in ¼ of the stained embryo (18/68). Genotyping performed in 20 embryos (short phenotype: n=12; long phenotype: n=8).

b. Whole-mount in situ hybridization with *hoxa11b, hoxd11a, hoxa13b* at 18hpf in embryos from an incross between *nr6a1a*$^{+/-}$*nr6a1b*$^{-/-}$ showing an extended anterior staining in *nr6a1a*$^{-/-}$ mutants.

c. Whole-mount is situ hybridization with *hoxa13b* at 24hpf in embryos from an incross between *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ double heterozygous. Classification of the embryo following the extend of the staining in the posterior mesendoderm (from absent to severely extended). Genotyping was performed for 19 embryos.

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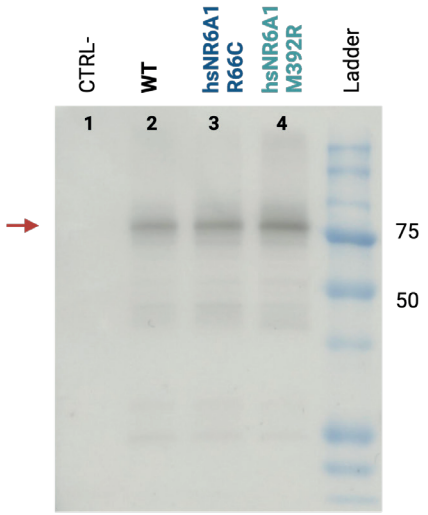
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Western blot after *in vitro* translation of wildtype and mutants NR6A1.

Western blot after in vitro translation showing similar concentration for the wildtype and mutants hsNR6A1. Ladder: Precision Plus Protein™ All Blue Prestained Protein Standards #1610373

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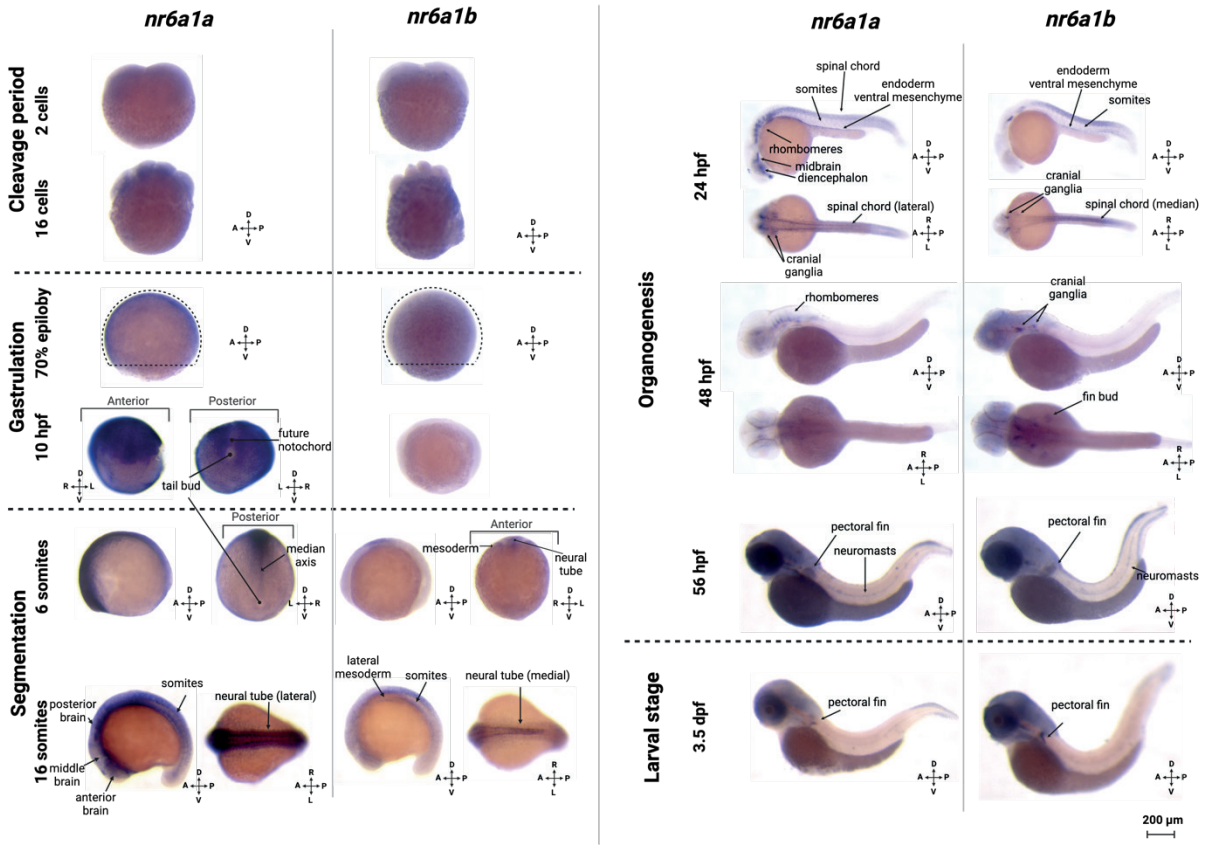

Supplemental Figure 2. Expression of nr6a1a and nr6a1b during embryonic development.

In situ hybridization at different stage of development for *nr6a1a* and *nr6a1b* on wildtype embryos.

Cleavage period: between 0 and 2 hpf, *nr6a1a* shows a mild ubiquitous expression, this early expression (before the maternal zygotic transition) supporting maternal expression of the gene in the oocytes. In contrast, *nr6a1b* is not detected at this stage. Pictures at the 2 cells (0.75 hpf) and 16 cells (1.5hpf) stage.

Gastrula period: At 70% epiloby (7hpf), *nr6a1a* is highly expressed ubiquitously in the gastrula while *nr6a1b* is not expressed. At 10hpf, an anteroposterior gradient is observed for *nr6a1a* with weaker signal in the posterior bud and the future notochord. The higher signal in the dorsal region is due to the higher cell mass in that region.

Segmentation period: At the 6 somites stage (12hpf), *nr6a1a* is expressed following an anteroposterior gradient, with a stronger signal in the anterior region and absence of expression in the tailbud. At this stage, *nr6a1b* starts to be expressed in the neural tube, paraxial and lateral mesoderm. At the 16 somites stage, *nr6a1a* is still highly expressed with strong signals in the ventral region of the anterior and midbrain, as well as in the

lateral part of the neural tubes and somites. *Nr6a1a* expression is lower in the posterior part of the embryo (tailbud). *Nr6a1b* is expressed in the region of the trunk in the neural tube, the somites and the lateral mesoderm.

Organogenesis: At 24hpf, *nr6a1a* expression is restricted to the ventral part of the diencephale and midbrain, the lateral part of the rhombomeres, the spinal cord, the cranial ganglia and in the endoderm/ventral mesenchyme. A mild expression is observed in the somites. *Nr6a1b* is expressed in the cranial ganglia and the spinal cord (median region). A mild expression is detected in the endoderm/ventral mesenchyme and in the somites. At 48hpf, the expression is limited to the rhombomeres for *nr6a1a* and the cranial ganglia and fin buds for *nr6a1b*. At 56hpf and 3.5 dpf (larval stage), the expression is barely detected and limited to the pectoral fin, the neuromasts and a structure (undetermined) posterior to the eyes for both genes.

A=anterior; P=posterior; D=dorsal; V=ventral; R=right; L=left

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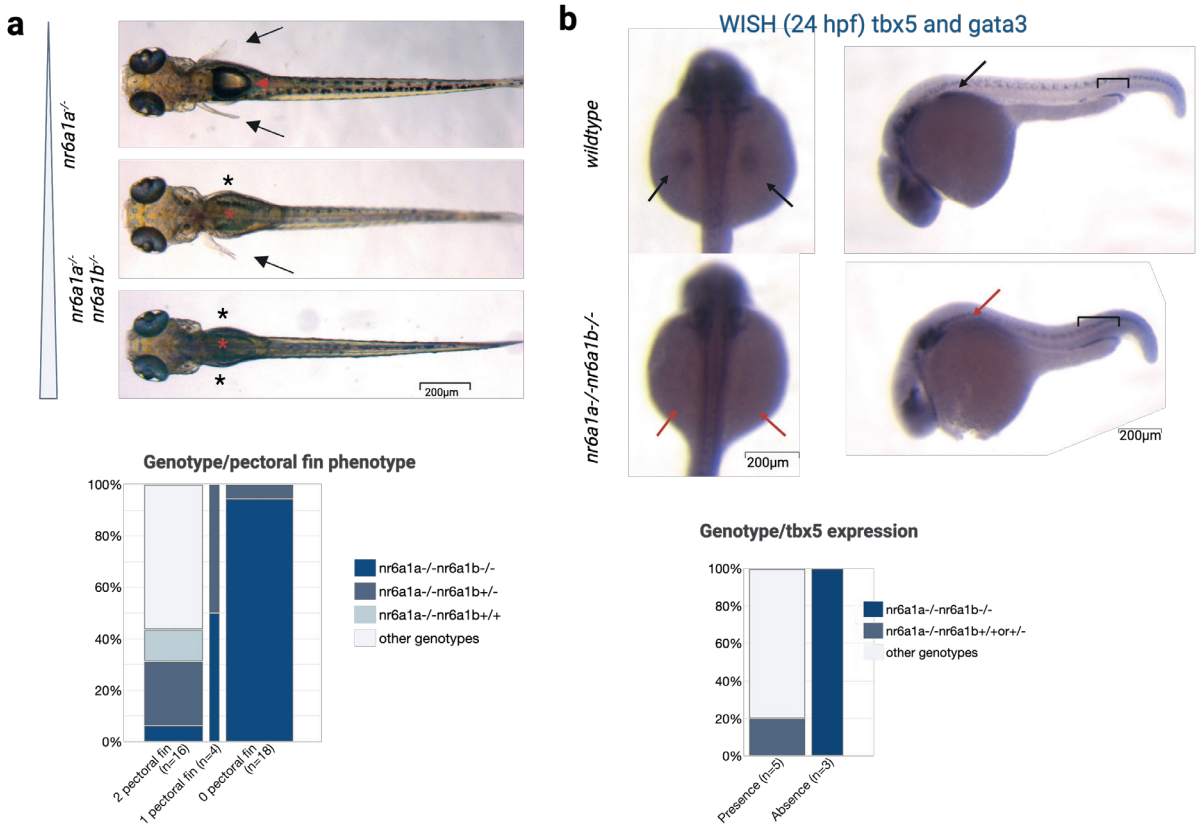
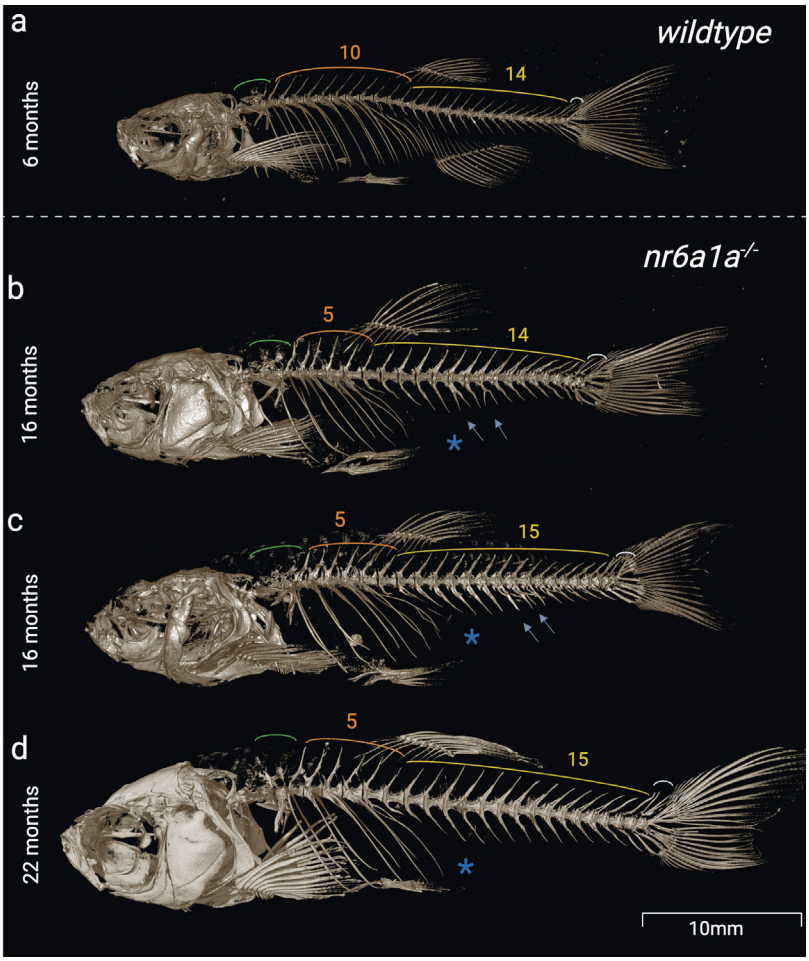


Supplemental Figure 3. Aplasia/hypoplasia of the pectoral fins in *nr6a1a*$^{-/-}$ and *nr6a1a*$^{-/-}$ *nr6a1b*$^{-/-}$ mutants.

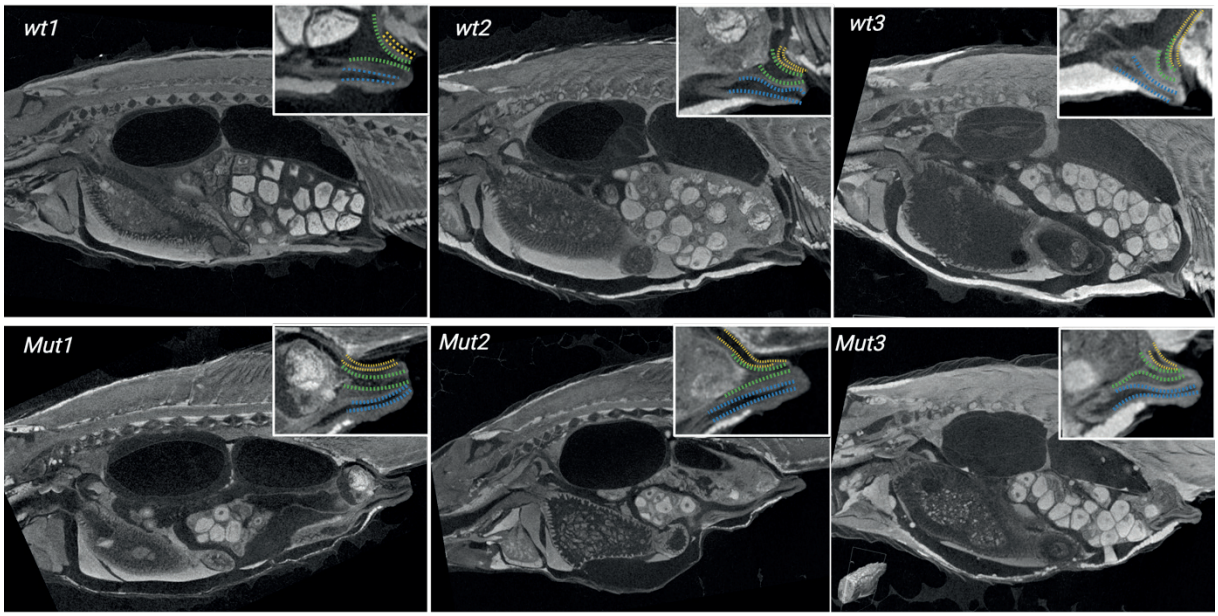
a. Dorsal view of 5dpf larvae form *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ incross. The pectoral fins are pointed by the black arrow (* when absent). Genotyping of 38 larvae showing that the majority of larvae without pectoral fins are double homozygous. 17/20 genotyped double homozygous larvae have no pectoral fin showing a high penetrance of the phenotype when both orthologues are knocked out. Absence of the swim bladder in affected larvae (red *) compared to the normal phenotype (red arrow).

b. Whole-mount in situ hybridization for *tbx5* and *gata3* in 24hpf embryo, showing absence of *tbx5* staining and extended *gata3* staining in 18/95 embryo from *nr6a1a*$^{+/-}$ *nr6a1b*$^{+/-}$ incross. Genotyping was performed for 8 embryos.



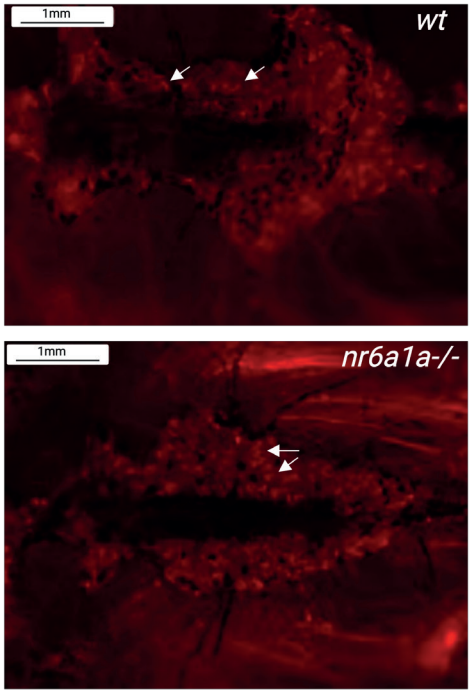
Supplemental Figure 4. X-ray microtomography of adult wildtype and mutants.

The number of rib-bearing precaudal vertebrae (orange) is decreased in the mutants (#5 instead of 10 in wildtypes). The rib-bearing precaudal vertebrae are recongizable by the presence of ribs, and the absence of hemal arches and spines. The number of caudal vertebrae (yellow, excluding the caudal fin vertebrae) varies in the mutants between 14 and 15 (number known to range from 14 to 16 in zebrafish with an average of 15 vertebrae (72) (Bird 2001). These vertebrae are recongnizable by the presence of hemal arches and spines. Malformed hemal arches that are unfused at the midline are observed in two mutants (b and c: blue arrow). The caudal fin vertebrae (white), that support the caudal fin rays, are normally composed of three vertebrae. The antepenultimate and penultimate vertebrae are not distinguishable in two mutants (b et d). Radials of the dorsal fin (unpaired median fin) begins between the 3rd and 5th neural spines of rib-bearing vertebrae in mutants and between the 9th and 10th neural spines of rib-bearing vertebrae in wildtype.The anal fin (beginning at the 1st caudal vertebrae in wildtype) is absent in mutants (*).The weberian vertebrae (vertebrae 1 to 4) are highlighted in green and seems normal although not investigated in details. Created with BioRender.com



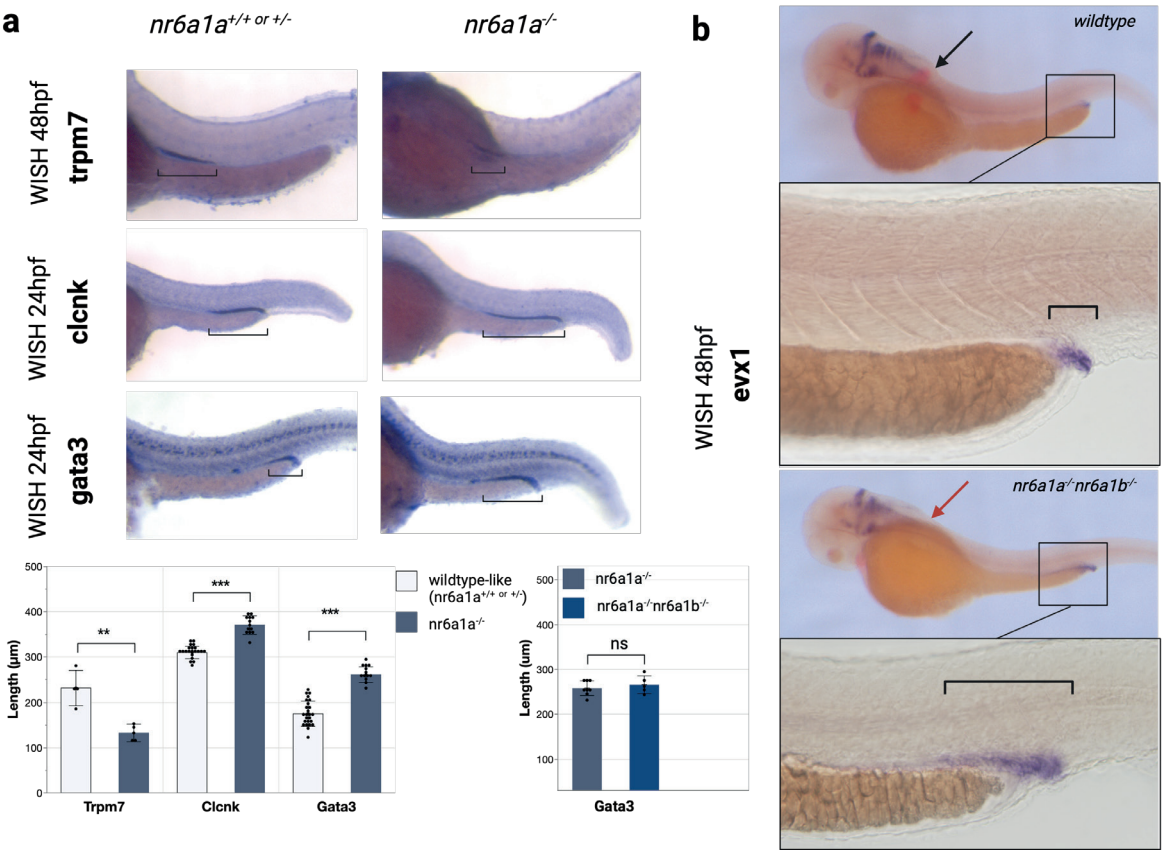
Supplemental Figure 5. Oviduct in *nr6a1a-/-* mutants

Micro-Ct scan after Lugol staining in 3 month-old wildtype (n=3) and *nr6a1a*$^{-/-}$ mutants (n=3).In mutants, the oviduct seems formed. The duct seems obstructed by degenerating oocytes.Created with BioRender.com



Supplemental Figure 6. Uptake of Rhodamine-Dextran seems normal in *nr6a1a*$^{-/-}$ mutants.

Rhodamine-dextran compounds seem normally incorporated by the epithelial cells in the proximal circumvulated tubule (red fluorescence, black arrow). Created with BioRender.com

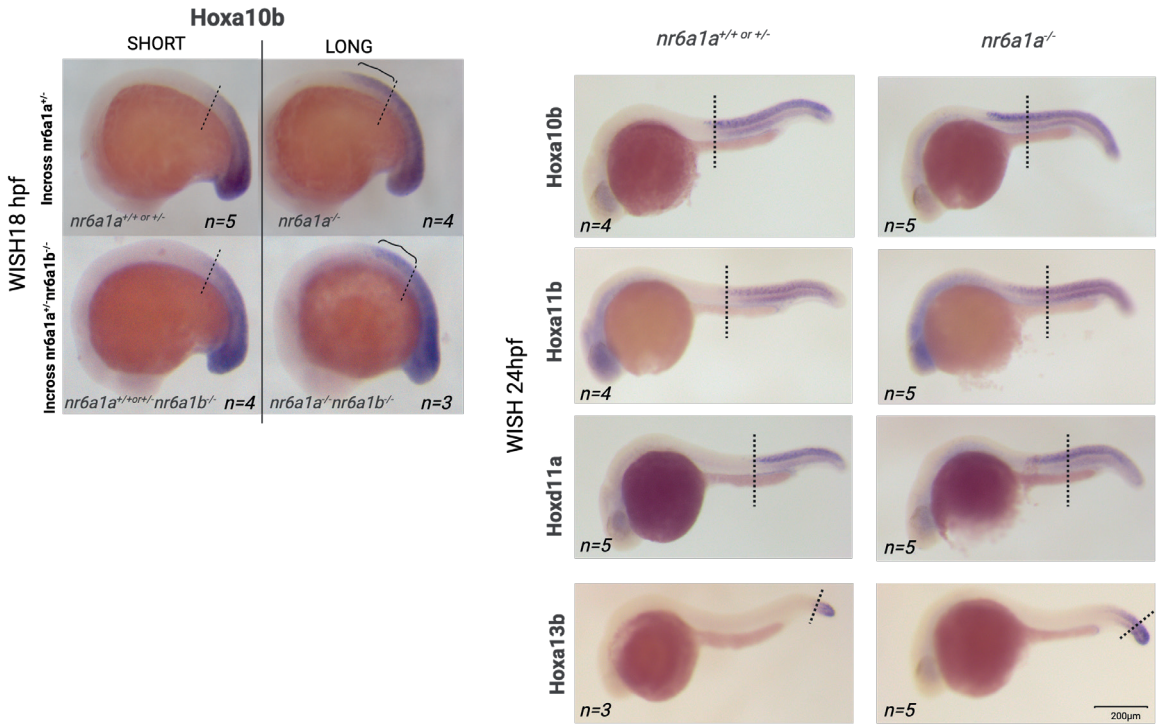


Supplemental Figure 7. Pronephros expression

a. Expression of segment markers was performed on embryos from *nr6a1a*$^{+/-}$ incross (*clcnk*) or *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ incross (*trpm7, gata3*) at 24hpf (*gata3, clcnk*), 48hpf (*trpm7*). Measurement (in µm) were statistically different between wildtypes and *nr6a1a*$^{-/-}$ for *trpm7* (n=8), *clnck* (n=33) and *gata3* (n=38). No statistically significant difference was observed for *gata3* between *nr6a1a*$^{-/-}$ (n=8) and double mutants (n=5).

b. Expression of *evx1* (coloration black NBT-BCIP) and *tbx5* (coloration fast-red) was performed on 89 embryos from *nr6a1a*$^{+/-}$*nr6a1b*$^{+/-}$ incross at 48 hpf. 8/89 embryos show no *tbx5* staining (previously shown to be associated with the mutant phenotype) and marked anterior extension of the *evx1* staining.

Lateral view of the embryos. Brackets indicate the expression domain in each embryo. Created with BioRender.com



Supplemental Figure 8. Abnormal patterning of posterior Hox in *nr6a1a*$^{-/-}$ and *nr6a1a*$^{-/-}$ *nr6a1b*$^{-/-}$ mutants.

a. Comparison of whole-mount is situ hybridization with *hoxa10b* at 18hpf in embryos from incross between *nr6a1a*$^{+/-}$*nr6a1b*$^{+/+}$ and embryo from an incross between *nr6a1a*$^{+/-}$ *nr6a1*$^{-/-}$. No significant difference is observed between *nr6a1b*$^{-/-}$ and wildtypes (SHORT phenotype). Extended anterior staining (LONG phenotype) in simple *nr6a1a*$^{-/-}$ and double *nr6a1a*$^{-/-}$*nr6a1b*$^{-/-}$ mutants.

b. Whole-mount is situ hybridization with *hoxa10b, hoxa11b, hoxd11a, hoxa13b* at 24hpf in embryos from an incross between *nr6a1a*$^{+/-}$*nr6a1b*$^{-/-}$ showing an extended anterior staining in *nr6a1a*$^{-/-}$ mutants.

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3. ADDITIONAL RESULTS

NR6A1 expression is induced by retinoic acid treatment in mouse and human embryonic stem cells (324,325) and *in vivo* in xenopus (326). Retinoic acid regulation of *nr6a1b*, and to a lesser extent of *nr6a1a,* was also previously shown in ChIP-seq data and RNA-seq data generated from endodermal tissues of 11.5hpf zebrafish treated with RA or DEAB (327), and confirmed by WISH on zebrafish embryo at different stages (Figure 22). As *nr6a1a* mutants show phenotype similarities with *aldh1a2* mutants, although less drastic (e.g., absence of pectoral fin, loss of pharayngeal arches, similar patterning defect of the pronephros)(302,328), we hypothesized that NR6A1 could be a downstream effector or modulator of retinoic acid signaling. Research to investigate a possible role of *nr6a1a/b* in the retinoic acid pathway is still ongoing under the lead of Bernard Peers in the ZDDM research lab and we will briefly expose the first results (Figure 23). WISH with the marker gene gata3 was performed on embryos of double heterozygous *nr6a1a/b* incross at 24hpf after treatment with retinoic acid (100nM) at the beginning of gastrulation (50% epiloby). After retinoic acid treatment, the gata3 staining was nearly absent in *nr6a1a* wildtype embryos, which indicates a severe anteriorization, as expected. Retinoic acid treatment of mutants leads to an intermediate staining for *nr6a1a*$^{-/-}$ (mildly shortened) whereas treatment of double mutants did not show any effect (long). These results suggest that both nr6a1a and nr6a1b are required for retinoic acid to promote a proximal fate, and that exogeneous retinoic acid treatment may attenuate the renal phenotype associated with loss of *nr6a1a*. In order to investigate if the repressive role of *nr6a1a/b* on gata3 expression in the pronephros depends on retinoic acid, embryo of single and double heterozygous *nr6a1a/b* incross were treated with DEAB (10µM). The anterior border of *gata3* staining expanded 1 somite rostrally in treated wildtype embryos (length of 4-5 somites) compared to untreated embryo (length of 3-4 somites). A longer staining (up to 7 somites) was observed in one quarter of the laying suggesting that these embryos were homozygous for *nr6a1a*$^{-/-}$ (Figure 23). If future genotyping confirms this correlation, the longer staining observed in DEAB treated mutants compared to DEAB treated wildtype would argue that *nr6a1a* represses *gata3* at least partially in an independent way from retinoic acid.

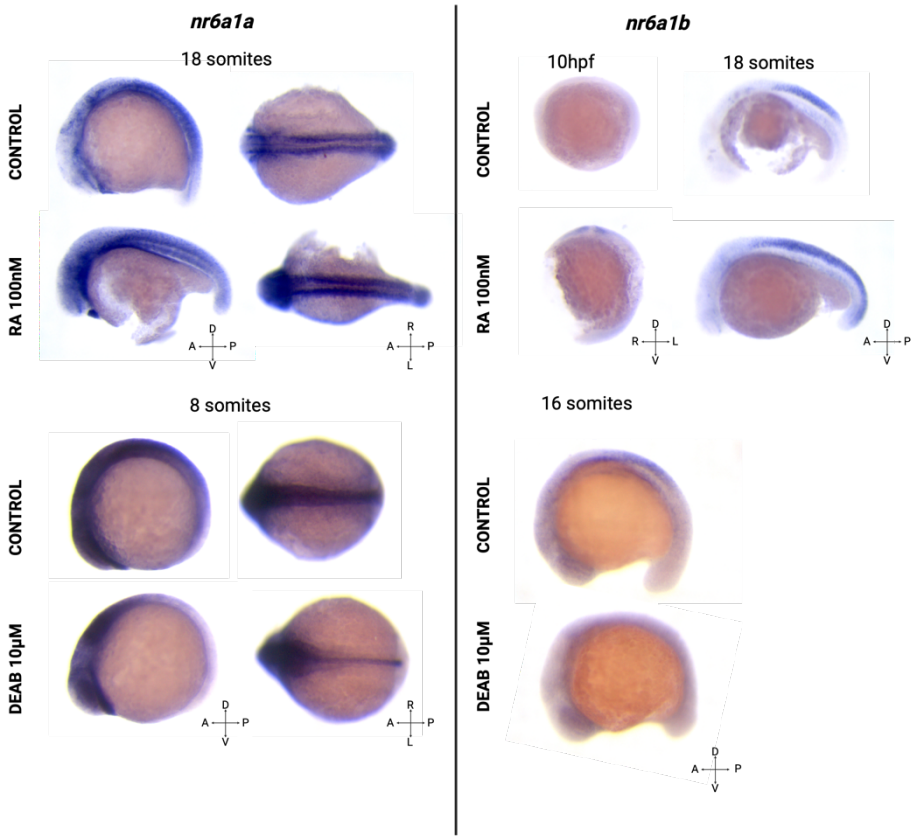

Figure 22. Expression of *nr6a1a* and *nr6a1b* after RA and DEAB treatment. Whole-mount in situ hybridization for *nr6a1a* and *nr6a1b* on wildtype embryos without (control) and after treatment with RA 100nM or DEAB 10µM, at different stages of development. Increased expression of *nr6a1a* was visible at the 18 somites stage after treatment with RA (no differences was noted at the 8 somites stage, data non shown). After treatment with DEAB, *the nr6a1a* expression is decreased in the posterior region of the embryo from the 8 somites stage. An increase in expression is noted for *nr6a1b* already at the end of gastrulation (10hpf) after RA treatment. Treatment with DEAB lead to an absence of *nr6a1b* expression at the 16 somites stage.

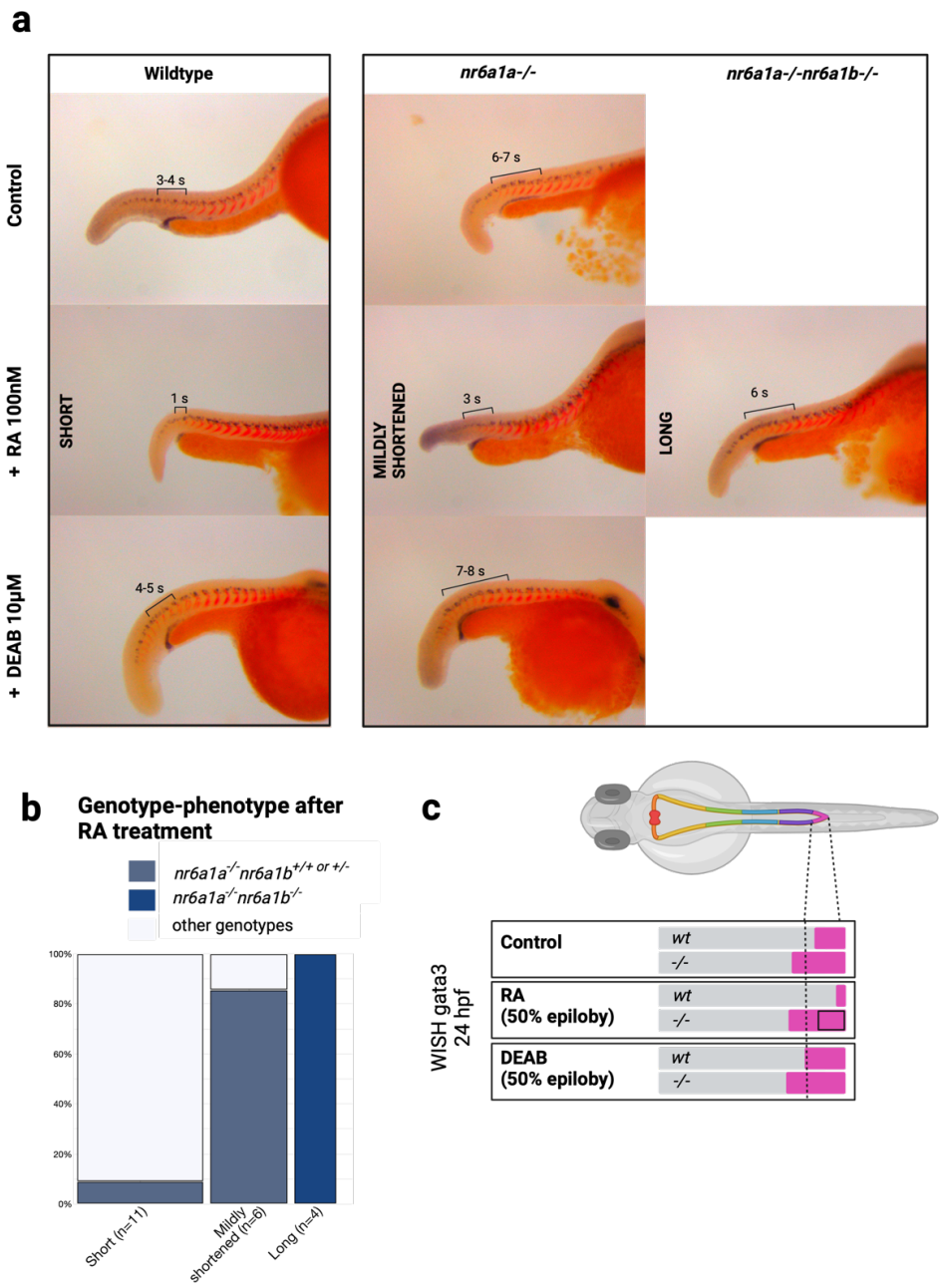

Figure 23. Nr6a1a/b are required for the action of RA on the distal segments.

a. Double whole-mount in situ hybridization with the somite boundary marker *xirp2a* and the pronephros segment marker *gata3* in embryo from *nr6a1a*$^{+/-}$ *nr6a1b*$^{+/-}$ incross in standard condition, after RA treatment at the 50% epiloby stage or after DEAB treatment at the 50% epiloby stage. The width of one somite is used as reference to measure the length of the expression domain. with the marker *gata3* at 24hpf, comparing the length of *gata3* staining (measured in somites number).

b. Genotyping performed in 21 embryos treated with RA.

c. Summary of the segmentation defect after RA and DEAB exposure.

CONCLUSION AND PERSPECTIVES

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The existence of multiple animal models and genetic syndromes featuring uterine malformations supports the notion of genetic heterogeneity in the etiology of congenital uterine anomalies, likely involving the interaction of multiple genes and molecular pathways, each contributing to uterine development and morphology. Allowing for the simultaneous analysis of multiple genes, whole exome sequencing offers a cost-effective and powerful tool for investigating genetic predisposing variants in selected families with recurrence.

In our first paper published in 2020, by analyzing nine families with recurrence of uterine malformations, we reported *GREB1L* as a significant gene in MRKH syndrome, contributing to the phenotype in four of nine families and up to 7.4% of sporadic cases in our series (2.9% when considering only variants predicted deleterious *in silico*). In 2023, in a larger series, Jolly et al. confirmed the involvement of *GREB1L* variants in MRKH, in a few but significant percentage (2.7%) of individuals (149). Whereas CAKUT are the most prevalent congenital defect associated with *GREB1L* variants, uterine, skeletal, hearing, cardiac and adrenals are other organ systems recurrently affected. Incomplete penetrance is frequently observed. Mechanisms by which *GREB1L* pathogenic variants lead to renal or uterine malformation are still poorly characterized, although abnormal tubulomorphogenesis was demonstrated in cells culture (30).

Conducting thorough analyses of exome data from our as yet unresolved families, we then identified the gene *NR6A1* as a novel candidate gene for renal and uterine malformations. Studies in zebrafish knocked out for the orthologous genes *nr6a1a* and *nr6a1b* confirmed a conserved and essential role for *NR6A1* during embryonic development and its involvement in kidney malformations. Modification in retinoic acid (RA) signaling/gradient and abnormal regulation of posterior hox genes expression by *NR6A1* may alter Müllerian duct elongation, as well as the processes of junction to the urogenital sinus and patterning of the female genital tract. Many questions remain regarding the regulatory mechanisms involving NR6A1 in renal and uterine development: is the mechanism of regulation direct and/or indirect (through modulation of the RA levels), what is the timing of action (early in development through fate specification, migration or differentiation of intermediate mesoderm progenitors, or by local expression

later on during development) and what other genes involved in renal and uterine development are regulated by *NR6A1*?

In the first part of this final section, we first address future research directions that will increase our understanding of the phenotypes linked to *NR6A1* and the role of the protein during renal and uterine development. We examine what is currently known about *GREB1L*, its function during development and some similarities between *GREB1L* and *NR6A1*. Finally, we discuss hypotheses on some pathways possibly involved in congenital uterine anomalies based on this work. In a second part, we explore potential explanations for the incomplete penetrance and variable expressivity that were observed in the nine families investigated here. Lastly, we examine the possible reasons for the absence of identified high-effect variants in four of the families.

I. FUTURE DIRECTIONS IN UNDERSTANDING NR6A1 AND GREB1L FUNCTIONS IN RENAL AND UTERINE DEVELOPMENT

1. DELINEATING THE NR6A1-ASSOCIATED PHENOTYPES AND ITS ROLE DURING RENAL AND UTERINE DEVELOPMENT

Delineating the NR6A1-related phenotypes

NR6A1 has emerged as a key player in elongation and patterning of the somites during the development of the trunk region in mice (329). The development of other trunk/abdominal organs is also impacted, as shown for the kidneys in our zebrafish model. Moreover, the diverse range of phenotypes observed in our zebrafish mutants suggest that the defects caused by loss-of-function of NR6A1 may variably extend beyond skeletal, renal and uterine malformation in human individuals also. Reports in the Genematcher database of several individuals with *NR6A1* variants and cardiac malformation support the hypothesis that defects in *NR6A1* may be associated with a broader phenotypic spectrum. Further studies in mice and zebrafish models, as well as sequencing of additional human cohorts, will be complementary to confirm the gene causality for renal agenesis and MRKH, and to gain better insight in the phenotypic spectrum associated with *NR6A1*.

First, in order to gain additional evidence for causality of *NR6A1* in renal agenesis and MRKH, investigation in a mouse model will be required. While our zebrafish model revealed an impact on kidney development and morphogenesis, it is limited by its inability to assess the more evolutionarily advanced stages of kidney development, such as ureteric bud outgrowth and metanephros formation, which are unique to amniotes. Notably, renal agenesis often results from disrupted signaling between the metanephros and the ureteric bud. Moreover, homologies between the gonadal duct, in zebrafish, and uterus in mammals are limited. Confirming the agenesis of kidney and uterus in mice will be necessary to get strong evidence for the gene causality. A conditional model will be required given that lethality occurs before the development of the metanephros and Müllerian ducts in knockout mice. Finally, sequencing of *NR6A1* in cohorts of patients with CAKUT/renal agenesis, and in wider cohorts of patients with MRKH syndrome, could help to identify additional human individuals with variants in the gene.

Second, we will perform a comprehensive phenotypic analysis of our zebrafish model to identify organs potentially affected by variations in *nr6a1a/b* dosage. Zebrafish offer several advantages over mice for this analysis, including lower costs, the ability to observe

hundreds of embryos per breeding *ex-utero*, and the availability of multiple transgenic lines that enable visualization of distinct organ development using fluorescent microscopy. These analyses of our zebrafish *nr6a1a/b* model will help screen for organs that may be impacted by *nr6a1a/b* loss-of-function or overexpression and assess their penetrance. Variable pathogenic mechanisms, such as loss-of-function versus gain-of-function, could result in distinct phenotypes, as demonstrated by skeletal defects observed in mice (329). In zebrafish, gain-of-function mutations can be characterized by overexpressing *nr6a1a* through microinjection of capped *nr6a1a* mRNA into one-cell stage embryos to identify the associated developmental defects. Additionally, generating knockin zebrafish with missense variants identified in humans will allow us to evaluate their effects and penetrance *in vivo*. Our mutant zebrafish also survive longer in development compared to knockout mice, especially when dechorionation is induced by adding pronase to their environment, as we observed that mutants exhibit hatching defects. Some double *nr6a1a/b* mutant zebrafish survive until 10 days post fertilization, and a small percentage of single mutants reach adulthood, whereas mouse embryos die after the time of the trunk-to-tail transition[10] at E10.5. This extended survival in zebrafish provides an opportunity to investigate organogenesis and later-occurring phenotypes. For example, further characterization of the ovarian phenotype will be particularly interesting, as our micro-CT scans suggest that mutants may experience precocious degeneration of oocytes. In mice, *Nr6a1* has been shown to be required for the maturation of primordial germ cells into developed oocytes (333). Hence, in humans, *NR6A1* is certainly an interesting candidate gene to sequence in cohort of individuals with premature ovarian failure.

Finally, given the phenotypic characteristics observed in our zebrafish mutants, individuals with VACTERL association represent another cohort of interest for investigating potential NR6A1-related phenotypes in humans. Notably, the zebrafish mutants exhibit agenesis or hypoplasia of the pectoral fins (analogous to the upper limbs in mammals) and cloacal anomalies (including absent gut opening), which mirror key features of VACTERL, such as radial ray defects and imperforate anus, along with the commonly observed renal and vertebral anomalies.

[10] The transitions from head-to-trunk and from trunk-to-tail, during embryonic development in vertebrates, involve major switch in the cellular and molecular mechanisms, each region of the body being regulated by distinct gene network. *NR6A1* has been shown to promote trunk growth and the gene is down-regulated at the trunk-to-tail transition. Key features of the trunk to tail transition are relocation of the neuromesodermal competent cell population, closure of the posterior neuropore, induction of the hindlimb and formation of the cloaca. The rostral border of the tail corresponds to the 21 somites in zebrafish (reached at 19.5hpf), and 28-30 somites in mammals (reached at E9.5-E10 in mice, day 27 in humans) (330–332).

Understanding the regulatory roles of NR6A1

NR6A1 belongs to the nuclear receptor superfamily of transcription factors[11]. In contrast to other members of this superfamily (e.g., steroid-hormone related receptors or receptors for retinoic acid), no ligand has been identified so far for NR6A1. In its constitutive state, the protein binds to the DNA consensus sequence DR0 $(AG(G/T)TCA)_2$ or to an extendend half-site (TCAAG(G/T)TCA), and recruits various corepressors complexes to repress the transcription of its target genes (Figure 24.a). During the development of the embryo, we confirmed that the expression of the gene is dynamic in zebrafish, similarly to mice, with a high level of expression during gastrulation and early segmentation stages (Figure 24.b), a lower expression in the most caudal region (tailbud) from the 6 somites stage onwards and very limited expression after 24hpf. These results support an essential role for *NR6A1* during gastrulation and early somitogenesis, a period corresponding to the ingression of the mesodermal precursors that will form the organs of the trunk. The signals and genes known to control the time-limited expression of *NR6A1* are key regulators of several developmental processes, including axial elongation (Figure 24.b).

Amongst the inducers of *NR6A1* expression is retinoic acid. We confirmed by WISH in our zebrafish model that *nr6a1a* and, even with a stronger effect, *nr6a1b,* are upregulated by exogeneous retinoic acid treatment, especially in the posterior paraxial regions. In the opposite, inhibition of endogeneous retinoic acid synthesis (by DEAB treatment of the embryo), decrease the expression of *nr6a1a* and totally abolish the expression of *nr6a1b*, showing a partial and total RA-dependent activation for *nr6a1a* and *nr6a1b* respectively.

[11] The nuclear receptor superfamily of transcription factors comprises 48 receptors in humans (49 in mice and 73 in zebrafish), that are evolutionary related and organized into seven subgroups based on sequence similarities. This superfamily includes, for instance, the steroid-hormone related receptors and retinoic acid receptors. Nuclear receptors share similar structural domains, including a highly conserved DNA binding domain (DBD) and a discrete ligand binding domain (LBD). They are able to regulate transcription of their target genes (either activating or repressing it) by binding to short DNA sequences in the promotor region or on a distal enhancer region, and interacting with coregulatory proteins that can interact with the RNA polymerase machinery or modify chromatin structure, thereby influencing gene accessibility. Their activity can be modulated by three-dimensional conformational changes triggered by ligand binding. These ligands include fat-soluble hormones, vitamins, or dietary lipids. Besides their natural ligands, nuclear receptors can also be inadvertently targeted by synthetic compounds, referred to as endocrine-disrupting chemicals, which may alter receptor activity and potentially lead to epigenetic modifications that could be passed on to subsequent generations (334). NR6A1 is the only member of subgroup 6 and is more closely related to proteins of the steroid hormone branch (335).

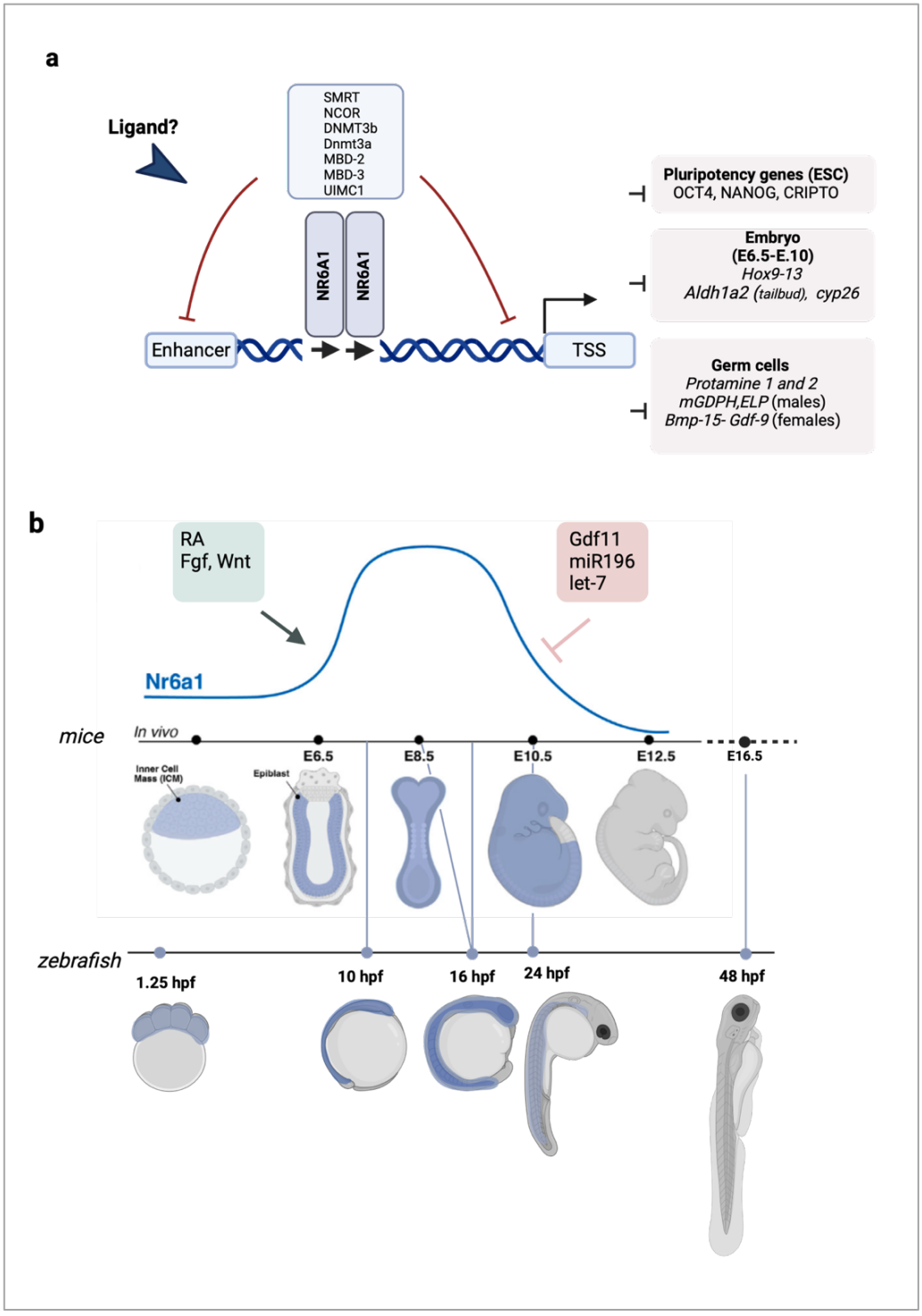

Figure 24. Expression and regulation of NR6A1.
NR6A1 binds as homodimers to the consensus sequence DR0 (depicted by black arrows), repressing gene expression of is target genes (in the gray boxes) through interaction with co-repressors (in the blue box). TSS= transcription start site. b. Dynamic expression of *NR6A1* based on *in situ* data in mice and zebrafish. Lines connecting the timelines represent correspondence between embryonic stages in zebrafish and mice (based on Irie 2011)(336). Picture adapted from Li 2024 (337). Created with Biorender.

Besides, *NR6A1* regulates the expression of key RA regulatory enzymes (ALDH1A2, CYP26) which support that *NR6A1* may partially regulate the retinoic acid gradient (172,326,338). In our zebrafish mutant, studies on pronephros segmentation (a process known to depend on the RA gradient as mentioned in the introduction (302)), suggest that *nr6a1a/b* may control the differentiation process of the distal and intermediate segments, repressing the most distal fate that specify the very last segment (named pronephric duct).

Furthermore, our experiments suggest that *nr6a1a/b* is required for the RA signal to be able to promote a more proximal fate on the most distal segment. In order to investigate when and how this patterning anomalies occur, we will pursue the analyses by *in situ* at earlier stages to delineate which markers of differentiation are affected, and to define the spatiotemporal alteration of *cyp26a* and *aldh1a2* expression in mutants. In mammals, retinoic acid signaling is required for the primary ureteric bud outgrowth (through regulation of *RET* expression), for branching morphogenesis, for proper insertion of the nephric ducts in the cloaca, for initiation and/or maintenance of the Müllerian ducts and for uterine patterning (18,70,243,303,339). Alteration or modulation in retinoic acid signaling is one hypothesis to investigate in order to better understand how loss of NR6A1 function could alter the normal development of the uterus.

As highlighted in our second paper, an alternative hypothesis to explain the renal, skeletal, and potentially uterine defects involves the disruption of posterior Hox gene expression patterns. Our zebrafish studies confirmed that NR6A1 plays a conserved role in regulating posterior Hox genes from groups A, B, and D. Specifically, we observed anteriorization of expression for hoxa/d9-13 and ectopic expression of hoxb13a in endodermal/mesodermal tissues. While the *hoxc* group was not yet investigated, we plan to perform *in situ* hybridization for *hoxc* paralogues, as recent findings have linked altered posterior *hoxc* expression to the absence of the anal fin (340). Anteriorization of posterior *hoxc* gene expression could thus explain the anal fin absence observed in our *nr6a1a/b*$^{-/-}$ mutants. Given that Hox genes are direct transcriptional targets of retinoic acid, the effect of NR6A1 on these genes could be indirect, potentially via modifications in retinoic acid availability, or direct, as suggested by predicted NR6A1 binding sites within posterior Hox genes (JASPAR database (https://jaspar.elixir.no/)) (341). Alterations in Hox gene patterning within the spinal cord and somites of our zebrafish mutants indicate that loss of *nr6a1/b* disrupts the establishment of the Hox code during the segmentation stage. In mice, somite precursors and the posterior intermediate mesoderm, which develop into the mesonephric and metanephric mesenchyme, originate from common Tbx6+ neuromesodermal progenitors located at the posterior end of the embryo (44). Given that

these tissues share a common origin, disruption of the Hox signature observed in somites suggests that a similar alteration could occur in the intermediate mesoderm, potentially interfering with differentiation processes or altering the spatial specification of cells among kidney and uterine precursors.

Additionally, NR6A1 may play a role in the subsequent differentiation of intermediate mesodermal cells. Single-cell transcriptomic data confirm *NR6A1* expression in the intermediate mesoderm, as well as in neuromesodermal progenitors and the caudal epiblast (Figure 25) (342). A later function in kidney and uterine morphogenesis is also plausible. NR6A1 is locally expressed in kidney cell lineages, such as the ureteric bud of human fetuses (10 to 18 weeks) (343) , and is differentially expressed in mouse cells transitioning from connecting duct cell types to collecting duct intercalated cells at E16.5-E17, suggesting its involvement in later stages of kidney development (344). The transcriptional landscape of the Müllerian duct during development has been studied in chicken, revealing NR6A1 transcripts without differential expression between the stages of duct formation and elongation (345).

Lastly, NR6A1 seems to have a wider action on late developmental genes (i.e., genes that are normally expressed after the trunk-to-tail transition), and likely repress other genes besides posterior hox genes. Precocious expression of other signaling molecules or transcription factors could impact kidney and uterine development. For example, Bmp7, predicted to be repressed by Nr6a1 (346), plays an inhibitory role in ureteric bud outgrowth onset (237). Dysregulated expression at critical times or locations may lead to renal agenesis. Furthermore, key genes involved in uterine and renal development, such as *Ret* and *Pax8*, are among the top 50 genes downregulated in *Nr6a1-/-* mice embryos in microarray transcriptomic analyses at E9.0-9.5 (329). Whether *NR6A1* directly regulates these genes through binding, or indirectly via pathways such as retinoic acid gradient modulation, remains to be elucidated.

In order to identify the genes targeted by *NR6A1* during embryonic development, we plan to perform ChIP-seq studies in zebrafish embryos, with a particular focus on genes involved in renal and uterine development. Complementary RNA-seq analyses in *nr6a1a/b* mutants and wildtypes embryo will help identify differentially expressed transcripts, and the key disrupted signaling pathways (e.g., retinoic acid (RA), Wnt, Fgf, and/or Bmp signaling). Additionally, comparing gene expression profiles between *nr6a1a/b* mutants and retinoic acid-deficient embryos will provide insights into shared and distinct regulatory mechanisms. If overlapping gene disruptions are observed, this would further support the role of *NR6A1* as modulator of the retinoic acid pathway.

2. THE LIMITED KNOWLEDGE ON GREB1L FUNCTION AND INTERACTION

Although previous mouse studies have shown that GREB1L loss-of-function impacts kidney and uterine development in mammals (30), the precise functions of GREB1L remain largely unknown. While characterizing the protein was beyond the scope of this work, it is worthwhile to explore hypotheses about its developmental role and highlight some parallels between *GREB1L* and *NR6A1*. Among the proposed functions of GREB1L, its potential interaction with the retinoic acid pathway is particularly compelling given RA's known role in renal and uterine morphogenesis. Like *NR6A1*, *GREB1L* transcription is significantly upregulated in RA-treated embryonic stem cells, suggesting that GREB1L may act as a downstream regulator of RA signaling (152). GREB1L has been shown to be part of a chromatin complex that includes its paralog GREB1, retinoic acid receptors (RAR/RXR), and estrogen receptors, suggesting a possible direct interaction with RAR/RXR receptors (153). Phenotypic similarities between *Greb1l* mouse mutants and embryos with partial RA deficiency, such as posteriorization of the fourth rhombomere, crisscross heart malformations, renal and uterine defects, further support a link between Greb1l function and the RA pathway (155). However, recent findings in mice suggest that while *Greb1l* mutants resemble partial RA deficiency, GREB1L does not directly alter RA signaling but rather affects parallel pathways involved in similar differentiation processes. Bernheim et al. observed that the retinoic acid response in *Greb1l* homozygous mutants was preserved in the cardiac region at E8.5, with unchanged expression of key RA-responsive genes and enzymes like *Aldh1a2* and *Rdh10* in the cardiac field (155). The published image shows a decrease of RA activity in the more posterior regions of the embryo, that the authors link to a phenotypic posterior truncation. Overall, the specific pathways and direct interactors of GREB1L remain to be elucidated.

Interestingly, *GREB1L* and *NR6A1* share notable similarities in their expression patterns and mechanisms of downregulation. Both genes are expressed at the end of gastrulation in posterior progenitor cells during trunk elongation, with *NR6A1* highly expressed in early neuromesodermal progenitors (E8.5) and *GREB1L* reported to mark mesodermal differentiation within these progenitors (156). Both genes are also upregulated in the presomitic mesoderm at E9.5 (346–348), and expressed in the intermediate mesoderm, peaking at E8.25 (342), suggesting their involvement in a regulatory network essential for trunk mesodermal differentiation or maintenance (Figure 25). Consistent with a role in mid-gestation embryonic regulation (E8.5-E10), both genes are repressed by the Let-7 family of microRNAs, which are highly expressed during

tail development (349). Moreover, both genes are upregulated in Gdf11 mutants, Gdf11 being a key player in the transition from trunk to tail regulatory networks (350). Finally, while segmentation and axial elongation have not been specifically studied in Greb1l mutants, the observed reduction in rib pairs (10 or 11 pairs) in three human fetuses with heterozygous *GREB1L* variants resembles defects seen in *NR6A1* mutants. This raises the possibility that GREB1L and NR6A1 may interact or participate in the same regulatory pathways during trunk elongation. Notably, *GREB1L* has been shown to be repressed by the protein OCT4 in embryonic stem cells prior to RA-induced differentiation (351), with *OCT4* itself being a well-known target of NR6A1 repression. Hence, loss of function of NR6A1 could indirectly lead to downregulation of *GREB1L* (Figure 25). Investigating potential changes in *greb1l* RNA expression in nr6a1a/b mutants would possibly provide further insights. Finally, since GREB1L has been reported as a putative nuclear receptor coregulator, it would be worthwhile to investigate potential direct interactions between the two proteins.

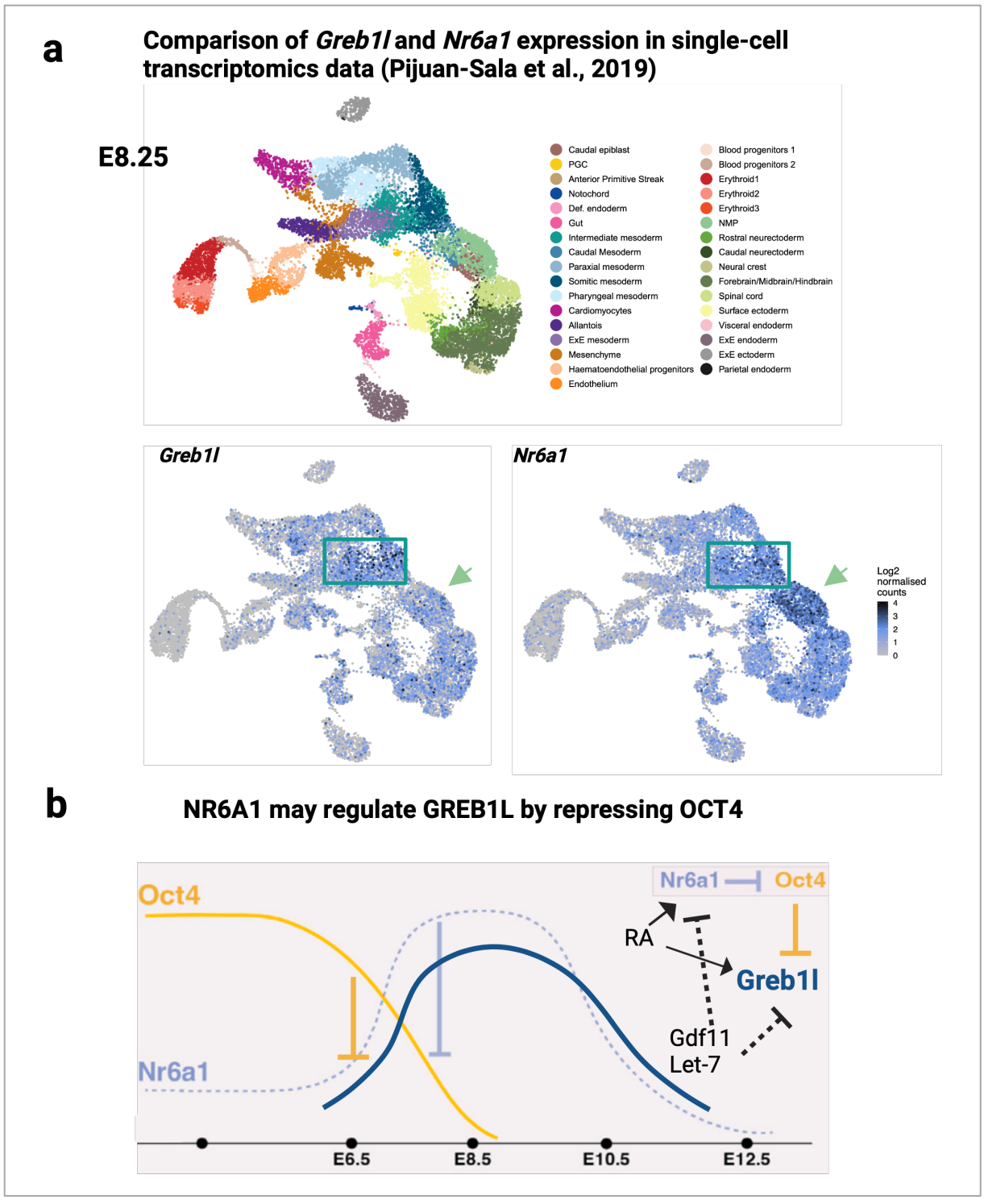

Figure 25. Similarities in Greb1l and Nr6a1 expression suggest that both proteins may be involved in the same regulatory pathway.
a. Expression of both genes at E8.25 in mice (From Pijuan-Sala 2019) (342). The green rectangle underlines intermediate mesodermal cells. The light green arrow point to neuromesodermal progenitors and caudal mesoderm cells that highly express *Nr6a1*.
b. Hypotheses on how NR6A1 may regulated GREB1L expression (figure adapted from Li 2024 (337) and created with Biorender.com).

3. HYPOTHESIS ON GENES/PATHWAYS INVOLVED IN CONGENITAL UTERINE ANOMALIES BASED ON THIS WORK

As outlined in the previous sections, further research is needed to clarify the functions of NR6A1 and GREB1L. Based on current knowledge, Figure 26 summarizes several hypotheses on potential molecular mechanisms that could explain uterine malformations in individuals with *NR6A1* or *GREB1L* heterozygous variants. Notably, our findings on NR6A1 suggest that the retinoic acid pathway (Hypothesis B) and posterior Hox genes (Hypothesis C) may play critical roles in uterine development. In humans, several genes associated with the RA pathway and posterior Hox genes are considered as potential candidates for MRKH syndrome or other uterine malformations. However, aside from *HOXA13*, current evidence supporting their causative role remains limited. Notably, a key distinction between *NR6A1* and other genes previously implicated (e.g., *HOXA10, RARA*) is that *NR6A1* broadly affects the patterning of posterior Hox genes rather than altering a single gene. Similarly, *NR6A1* may modulate the activity of various retinoic acid receptors in the posterior trunk region, either by upregulating the CYP26 degrading enzymes or by facilitating the function of multiple RAR/RXR receptors. Redundancy among HOX paralogues and RAR receptors may explain why variants in a single gene rarely result in malformations. In mouse models, significant effects on uterine morphogenesis were observed only when multiple Hox or RAR/RXR receptors were deleted. Thus, *NR6A1* defects could be more detrimental as they simultaneously affect the transcription of multiple Hox genes and broadly impact retinoic acid activity.

An additional comment on these two hypotheses comes from the type of uterine malformation and their putative embryological origin. The uterine malformations (hemi-uterus, uterine agenesis) observed in the majority of individuals with heterozygous variants in *NR6A1* and *GREB1L*, would result from defects in initiation or elongation of the Müllerian ducts, rather than defect in subsequent stages of differentiation. Such defects have been reported in mice knockdown for multiple Rar receptors, which support the importance of the RA signals for the formation or elongation of the ducts. In contrast, deletion of multiple posterior hox paralogous in mice, or ectopic expression of hox13 paralogues, result in differentiation defects (i.e., morphological and epithelial posteriorization or anteriorization), rather than in the absence of the uterus. To date, there is no evidence of an early Hox code in the Müllerian ducts, differential signature along the anteroposterior axis being detected after E14.5 in mice (i.e., after the connection of the ducts to the urogenital sinus). Although alteration of the hox pattern can easily explain the skeletal defects, and may explain the renal agenesis (by abnormal spatial expression

of hox11 paralogues), it is more difficult to link these alterations to the uterine phenotype of MRKH. We can though notice that *Hoxa13*$^{-/-}$ mice embryo have an absence of the caudal part of the Müllerian ducts at E14.5, which may suggest that the most posterior hox have a role in the rostro-caudal elongation of Müllerian ducts (63). Whether the ectopic expression of *hoxb13*, that we observed in our zebrafish mutants, may affect the caudal elongation of the Müllerian ducts may warrant further investigation in mice.

Finally, *GREB1L* and *NR6A1* may be part of regulatory network(s) involved in cell differentiation and specification (Hypotheses A and D), which is suggested by variation in their expression during embryonic stem cells, neural or mesodermal differentiation. Hence, loss of *GREB1L* or *NR6A1* may decrease the pool of mesonephric/mesoepithelial cells progenitors or their specification, affecting the initiation or elongation stages of the Müllerian ducts.

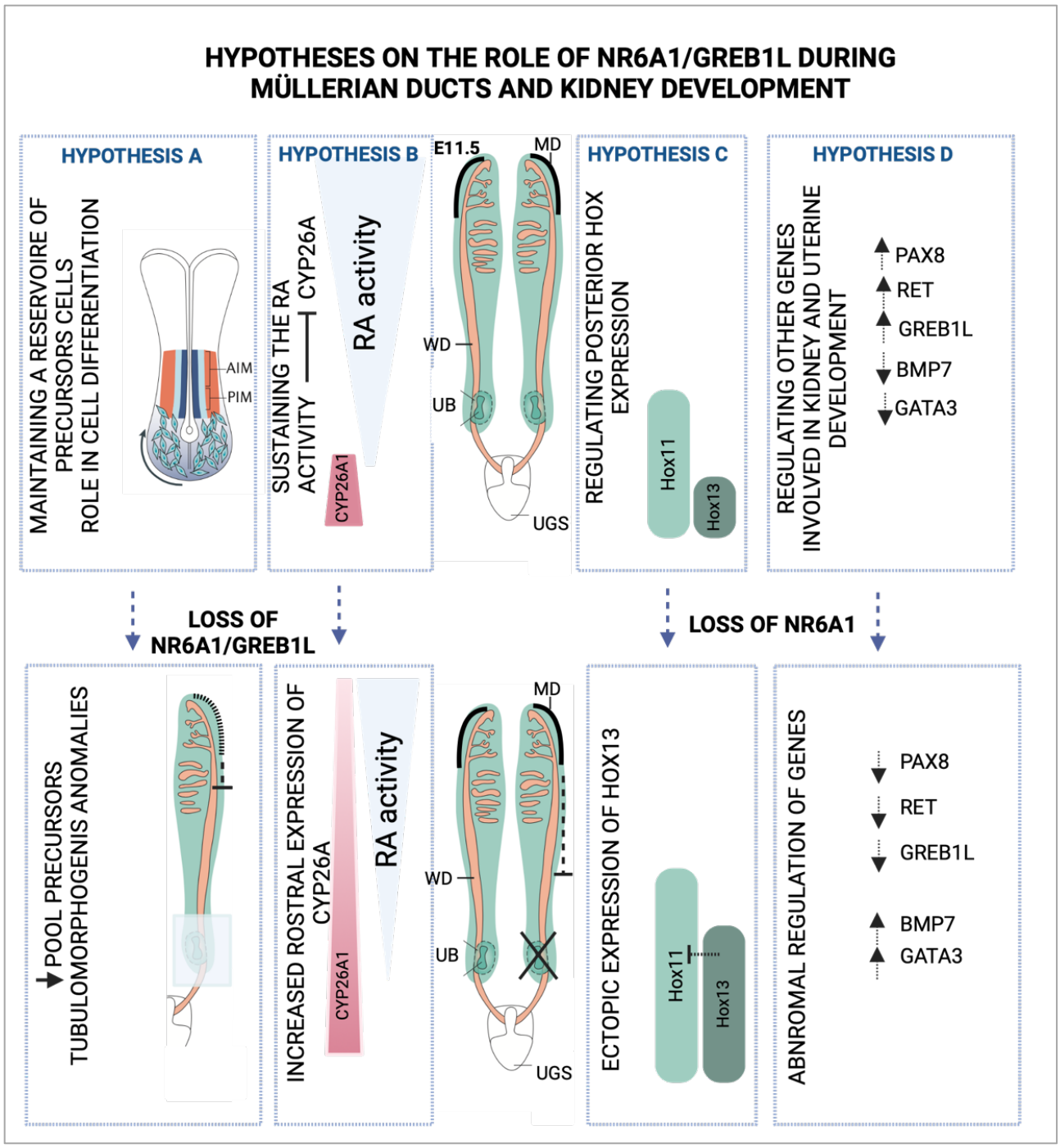

Figure 26. Potential molecular mechanisms that could explain uterine malformations in individuals with *NR6A1* or *GREB1L* heterozygous variants.
(the illustrations depicting the anterior and posterior intermediate mesoderm, as well as the development of the kidney system at E11.5 are from Short, 2016 (74)).
Created with Biorender.com

II. EXPLAINING INCOMPLETE PENETRANCE AND VARIABLE EXPRESSIVITY IN FAMILIAL CASES

In our analysis of nine families with uterine and renal malformations, we found that these malformations often follow a pattern of dominant inheritance with incomplete penetrance. Segregation analyses of families with identified *GREB1L* variants confirmed this pattern, as some unaffected relatives were heterozygous carriers. This inheritance model is frequently seen in monogenic causes of autosomal dominant CAKUT. Additionally, we observed variable expressivity in both *GREB1L* and *NR6A1* families. In this section, we will explore several factors that may explain the incomplete penetrance and variable expressivity, including genetic modifiers, stochastic variations in spatiotemporal gene expression, epigenetic differences, and environmental influences. While a strong genetic predisposition may be present, these additional factors might be necessary to reach the clinical threshold for disease manifestation.

Rare or common genetic modifiers

Incomplete penetrance can be explained by the presence, in one individual, of additional rare or common genetic modifiers that can increase the risk. The variants may be in other genes (model of digenic, oligogenic or polygenic inheritance) or in the same gene, affecting the second allele in a hypomorphic manner (referred as the compound inheritance gene dosage model (352)) . This hypomorphic variant can be frequent in the population and/or non-coding as seen, for instance, with *TBX6* (discussed in the introduction section). In the oligogenic and polygenic models, the cumulative effect of low frequency or common SNPs affecting the expression of the wildtype allele, or expression of genes in related pathways, might modify the penetrance (353). Finally, the genetic modifiers may also be protective, and then, present in unaffected carriers.

In our analysis of the solved familial cases, we evaluated additional rare variants in the exome data of the most affected relatives in Family 7 and 9 to explain the incomplete penetrance and variable expressivity (Supplemental table 4 of the first publication). However, we could not identify any likely pathogenic variants and faced challenges in selecting potential genes of interest due to limited knowledge on genes and pathway interactions for *GREB1L* and *NR6A1*. We did not investigate variants located in non-coding regions, as these were not captured by exome sequencing, and limited the analysis to rare variants that have a MAF <0.5%. Sequencing the entire gene and regulatory regions through long-read sequencing in affected and unaffected, or mildly affected, carriers of *GREB1L* or *NR6A1* variants would be interesting to investigate if recurrent

hypomorphic variants could contribute to the variable penetrance and expressivity in these conditions.

Finally, we did not investigate common SNPs in other genes that could contribute to differences in penetrance and expressivity. Although a few SNPs have been associated with uterine malformations or CAKUT (354–357), these findings await replication, and the implicated genes have not been directly linked to *GREB1L* or *NR6A1*.

Stochastic modification in gene expression

While genetic background and environmental exposure can explain interindividual variability, they do not account for phenotypic discordance in uterine or renal anomalies between monozygotic twins or intra-individual variability. Intra-individual variability—where one side of the body is affected differently than the other, such as unilateral renal agenesis or hemi-uterus—was observed in the families studied and is commonly reported in CAKUT. A plausible model to explain this variability is the Random autosomal monoallelic gene expression (RME), which refers to the stochastic transcription of a gene from one of two homologous alleles. During development, RME is thought to fine-tune regulatory pathways by dynamically and tissue-specifically regulating gene expression at critical times. RME can result in the random expression of one of two functionally distinct alleles (i.e., one wildtype, and one carrying a loss-of-function variant), leading to variations in the levels of functional protein—from normal levels to complete absence—in a critical subset of cells. This mechanism has been demonstrated for dosage-sensitive genes like the transcriptional co-factor *EYA1* (associated with Branchio-oto-renal syndrome) and the transcription factor *TBX5* (associated with Holt-Oram syndrome), potentially explaining phenotypic heterogeneity in these conditions (358,359). Similarly, local fluctuations in functional *NR6A1* and *GREB1L* proteins due to RME could result in variable phenotypic outcomes in heterozygous carriers or differential effects on each side of the body. Additionally, random variations in mRNA levels of other genes involved in the same regulatory pathways could shift the balance, influencing whether the pathogenic threshold is reached in one individual but not another, or asymmetrically between sides of the body.

Epigenetic modifiers

Incomplete penetrance between individuals with the same heterozygous variant may also be due to differences in the mRNA levels secondary to epigenetic variations in DNA

methylation, histone modifications or due to variation in microRNA (miRNA)[12] expression (127). For instance, several microRNA were shown to significantly regulate the expression of *NR6A1*. Amongst these, the miR-196 family (their genes being located on the hoxa;b and c clusters, and conserved in vertebrates) downregulate *NR6A1* expression in the tailbud at the trunk-to-tail transition (337), the 3'UTR region of NR6A1 harboring multiple binding sites for miR-196 genes. An antagonist relationship is also suggested from previous studies in zebrafish, showing overexpression of miR-196 phenocopies *nr6a1a/b* null mutant in regards to the pectoral fin phenotype, and the reduced number of ribs (360). Hence, overexpression of the micro-RNAs miR-196 may increase penetrance or severity of the phenotype in *NR6A1* heterozygous by decreasing the dosage of NR6A1 below 50%. Mi-RNA are regulated by DNA methylation (of their promotor or promotor of their host genes) but also by endogeneous (e.g., hormones) or exogeneous factors (e.g., hypoxia) (361) and can be inactive due to single nucleotide variations. Epigenetic variations may be both tissue-specific and time-dependent, complicating the understanding and detection of how epigenetic mechanisms influence the expression of the phenotypes (127). Notably, while epigenetic differences can sometimes be inherited, they can also result from environmental influences (e.g., effect of malnutrition, vitamin deficiency,..).

Environmental factors

Another explanation for the incomplete penetrance pattern lies in the interplay of genetic and environmental factors, suggesting that both predisposing genetic elements and environmental exposures during pregnancy may be necessary to reach a threshold that disrupts one or more developmental pathways. Evidence from animal studies and human teratogenicity highlights the significant role of environmental factors in uterine development. For example, agenesis or incomplete development of the Müllerian ducts has been observed in fetuses of pregnant rats fed with a vitamin A-deficient diet (362), while exposure to the endocrine disruptor diethylstilbestrol (DES) has been linked to the development of malformed T-shaped uteri in daughters of treated women, as already discussed in the introduction. Genetic predisposition may make certain individuals more susceptible to environmental influences. We could hypothesize that a decrease in vitamin A (and consequently retinoic acid levels) might increase the risk of renal and

[12] Micro-RNA are small non coding RNA (18-22nt) that regulate the stability or translation of mRNA by binding to their 3'UTR. One miRNA can influence multiple genes, and a gene can be affected by several miRNAs, potentially highlighting how variants in one miRNA may lead to multiple downstream phenotypic effects (127)

uterine defects in fetus with heterozygous variant in *NR6A1* or *GREB1L*, by downregulating the expression of the genes, or through a synergistic effect on retinoic acid availability. Conversely, vitamin A supplementation could potentially help prevent CAKUT or uterine malformations in these fetuses. Given the possibility to control their environments, zebrafish are valuable models for investigating how chemicals or environmental changes impact development. Preliminary results suggest that exposure to exogenous retinoic acid in *nr6a1a* -/- mutant embryos may partially alleviate their pronephros patterning defects. Further studies are needed to confirm these findings and identify which phenotypes might be improved through retinoic acid administration.

Moreover, since NR6A1 is a nuclear receptor, it is possible that its activity could be modulated by chemical ligands, similar to what has been demonstrated for other nuclear receptors (363). Endocrine disruptors may not only alter the transcriptional activity of these receptors during exposure but also induce epigenetic modifications that could have lasting impacts, potentially affecting subsequent generations if they influence primordial germ cells.

Enhancing our understanding of the environmental factors contributing to uterine malformations could inform the development of preventive measures. Concurrently, advancing our knowledge of genetic contributors can help identify individuals who may be more susceptible. Future potential prevention strategies might include banning exposure to specific endocrine disruptors, or recommending vitamin supplementation. However, it is essential to approach vitamin A supplementation cautiously due to the potential teratogenic effects of excessive retinoic acid. Additional research is required to deepen our understanding of the genetic and environmental factors that may elevate risk in genetically predisposed individuals.

III. UNSOLVED FAMILIAL CASES

Our study identified a definitive disease-causing variant in three families, which is quite a high yield (30%) compared to previous analyses in cohorts of individuals with MRKH/CUA. It also led to the discovery of a novel candidate gene (*NR6A1*) requiring further research to validate its causality in congenital uterine anomalies. This highlights the interest to focus research on multiplex families.

However, we were unable to identify a causative variant in four of the families. Several factors may contribute to this, as discussed in the introduction. First, limited understanding of gene function and the absence of relevant animal models may have led us to overlook potential causal genes or variants. Second, the inheritance pattern in these families might be oligogenic, involving multiple frequent variants, whereas our study was not designed to capture variants with a frequency greater than 1%. Moreover, exome sequencing limits the detection of rare variants in non-coding regions, and chromosomal microarrays may miss small deletions or complex chromosomal rearrangements. Finally, the small size and number of affected relatives in some families may complicate data analysis. For instance, interpreting results in Family 3 was challenging due to the high number of shared heterozygous variants between the two affected sisters. Additionally, the hypothesis of incomplete penetrance—common in CAKUT—prevented us from using unaffected family members to rule out certain variants.

Another approach for investigating these unresolved families in the future could involve whole-genome sequencing, which would enable the examination of non-coding regions. Additionally, utilizing long-read sequencing rather than short-read sequencing would provide advantages for detecting structural variants and for conducting methylation analysis.

IV. CONCLUSION

In conclusion, this study reinforces the role of genetic factors in congenital uterine malformations and highlights the significant benefits of whole exome sequencing in multiplex families with recurrent congenital uterine and renal anomalies. We provide new evidence supporting the causality of *GREB1L* in MRKH syndrome and identify *NR6A1* as a candidate gene, broadening the genetic landscape of this condition. Further sequencing in additional cohorts will be crucial to better delineate the phenotypic spectrum of these genetic disorders. Genetic counseling for affected families remains challenging due to the incomplete penetrance and variable expressivity of these genes, which complicates the prediction of outcomes for heterozygous offspring. While we would not recommend prenatal diagnosis in the absence of severe malformations, couples may consider preimplantation genetic testing when causality is well-established. Strengthening the evidence for NR6A1 involvement is thus important. Further studies are essential to clarify the functions of both genes and the mechanisms by which they contribute to uterine malformations. A deeper understanding of the pathophysiological mechanisms associated with these genes could also ultimately guide the development of preventive measures for at-risk pregnancies.

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APPENDIX

APPENDIX

Article: Jacquinet A, Millar D, Lehman A. 2016. Etiologies of uterine malformations. Am J Med Genet Part A 9999A:1–32.

RESEARCH REVIEW

AMERICAN JOURNAL OF medical genetics PART A

Etiologies of Uterine Malformations

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Ranging from aplastic uterus (including Mayer–Rokitansky–Kuster–Hauser syndrome) to incomplete septate uterus, uterine malformations as a group are relatively frequent in the general population. Specific causes remain largely unknown. Although most occurrences ostensibly seem sporadic, familial recurrences have been observed, which strongly implicate genetic factors. Through the study of animal models, human syndromes, and structural chromosomal variation, several candidate genes have been proposed and subsequently tested with targeted methods in series of individuals with isolated, non-isolated, or syndromic uterine malformations. To date, a few genes have garnered strong evidence of causality, mainly in syndromic presentations (*HNF1B, WNT4, WNT7A, HOXA13*). Sequencing of candidate genes in series of individuals with isolated uterine abnormalities has been able to suggest an association for several genes, but confirmation of a strong causative effect is still lacking for the majority of them. We review the current state of knowledge about the developmental origins of uterine malformations, with a focus on the genetic variants that have been implicated or associated with these conditions in humans, and we discuss potential reasons for the high rate of negative results. The evidence for various environmental and epigenetic factors is also reviewed.

Key words: uterus; Müllerian ducts; genes; congenital abnormalities; embryonic development

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INTRODUCTION

Uterine malformations include a broad spectrum of congenital anomalies arising from defects in formation, fusion, or septal absorption of Müllerian ducts (Fig. 1). Formation of Müllerian ducts (also known as paramesonephric ducts) begins with invagination of the coelomic epithelium within the intermediate mesoderm around the 6th week of embryogenesis gestation. The Müllerian ducts then elongate to reach the urogenital sinus around the 10th week. Thereafter, in *Homo sapiens* and other higher primate species, Müllerian ducts differentiate and fuse, forming a single uterus with a single cervix and vagina after complete regression of the midline septum around the 20th week. Müllerian ducts give rise to the fallopian tubes, the uterus, the cervix, and the inner part of the vagina [Hashimoto, 2003; Kobayashi and Behringer, 2003; Breech and Laufer, 2009; Massé et al., 2009; Connell et al., 2013]. Furthermore, Müllerian duct development proceeds in close physical and temporal proximity to the Wolffian ducts [Guioli et al., 2007], which informs the frequent association of renal and urological malformations in patients with Müllerian ducts anomalies (MDA) [Hall-Craggs et al., 2013a; McGowan et al., 2015; Rall et al., 2015b]. Formed by mesenchymal–epithelial transition in the intermediate mesoderm and reaching the cloaca at the 5th week, the Wolffian ducts (or mesonephric ducts) give rise to the ureteric buds in their caudal part as a result of inductive signals from the metanephros mesoderm. Orchestrated signaling between the metanephros and the ureteric buds is essential for normal development of definitive kidneys and excretory systems [Uetani and Bouchard, 2009]. Moreover, although Wolffian ducts ultimately regress almost completely in females, they are essential during development for processes such as Müllerian duct elongation [Chiga et al., 2014].

Several classification systems (Table I) have been proposed for the description of uterine anomalies or other female genital tract (FGT) malformations, for the purpose of guiding diagnosis and therapy [Oppelt et al., 2005; Buttram and Gibbons, 1979; Acién and Acién, 2011; Grimbizis et al., 2013]. The European Society of

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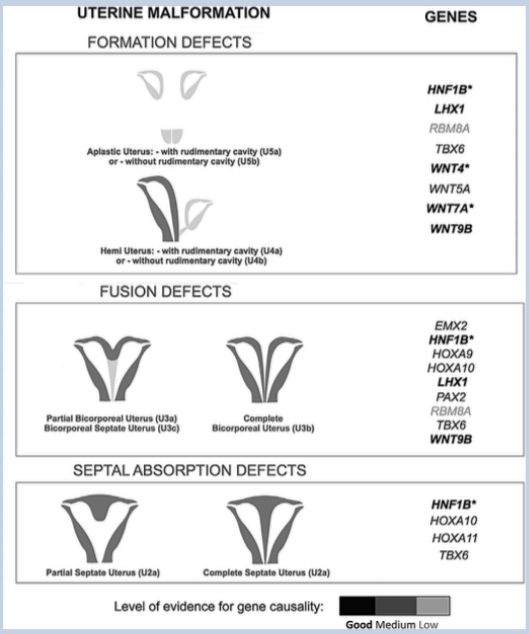

FIG. 1. Genes associated with isolated uterine phenotypes in humans. Uterine malformations are described following ESHRE/ESGE classification and are grouped according to the three stages of uterine development: formation, fusion, and septal absorption. Some genes are associated with multiple phenotypes. Gene names are bolder or lighter relative to the amount and quality of evidence for their involvement in uterine malformations (strongest with recurrence of rare and predicted damaging variants in affected individuals) and experimental data (strongest with supportive expression data, functional analyses, and/or mouse models showing involvement in uterine development and malformations) [MacArthur et al., 2014]. *Only one case associated with an isolated phenotype, otherwise associated with syndromic presentations.

Human Reproduction and Embryology/European Society for Gynaecological Endoscopy (ESHRE/ESGE) classification system groups uterine malformations according to the anatomical components and their corresponding embryological origins, reflecting defects in formation, fusion, and septal absorption [Grimbizis et al., 2013]. Cervical and vaginal malformations are also classified in this system, but as independent co-existent subclasses, reflecting the fact that these anomalies can be either isolated or manifest in various combinations with different uterine anomalies. Various cervical and vaginal defects, similar to uterine anomalies, may also arise in discrete developmental stages [Grimbizis and Campo, 2010]. This review does not comprehensively examine non-uterine Müllerian anomalies or all anomalies of the FGT but instead focuses on causes of uterine anomalies (with or without associated anomalies), as described in the ESHRE/ESGE classification. There are several other important Müllerian anomalies and FGT malformations, such as isolated cervical aplasia (also termed cervix atresia), vaginal aplasia (also termed vaginal atresia), transverse or longitudinal vaginal septum or imperforate hymen; and the etiologies of these anomalies are not specifically broached in this review.

Uterine malformations occur within a spectrum of variable expression and severity, the most severe end being absence of any fully or unilaterally developed uterus (variably termed aplastic uterus, uterus aplasia, uterine agenesis, or congenital absence of the uterus; class U5 in ESHRE/ESGE classification), with or without rudimentary cavities (unilateral or bilateral horns/uterine remnants). An aplastic uterus is usually diagnosed during late adolescence because of primary amenorrhea, with or without pain. Other uterine malformations include hemi-uterus (unicornuate uterus, uterus unicornis; class U4 in ESHRE/ESGE classification), partial or complete bicorporeal uterus (also referred in the literature as bicornuate or bifid uterus) and bicorporeal septate uterus (class U3 in ESHRE/ESGE classification), and partial or complete septate uterus (class U2 in ESHRE/ESGE classification). These anomalies may lead to medical or obstetric complications such as miscarriage, prematurity, fetal malposition, dystocia, low birth weight in offspring, or acute abdominal pain (e.g., from hydrometrocolpos), when they are associated with any cervical or vaginal obstructive anomaly [Dietrich et al., 2014; Hiersch et al., 2015]. Many uterine malformations may be totally asymptomatic and undiagnosed unless they are specifically sought. Frequently, these uterine malformations are also associated with anomalies of the vagina, cervix, fallopian tubes, or kidneys. Some of these associations have been described under specific terms and referred to as syndromic conditions. Mayer–Rokitansky–Kuster–Hauser syndrome (MRKH; MIM 277000; U5C4V4 in the ESHRE/ESGE classification) is characterized by congenital absence of the uterus, cervix, and the upper part of the vagina in an otherwise phenotypically normal 46,XX female. Fallopian tubes are frequently normal but may be hypoplastic/aplastic or malformed [Oppelt et al., 2012]. Persistence of at least the fimbriated end of the fallopian tube is typical and unexplained to date. The structure and function of the ovaries are usually normal although gonadal dysgenesis and ovarian agenesis have been described in a few cases [Rall et al., 2015b]. Ectopic ovaries have been reported in up to 40% of individuals [Hall-Craggs et al., 2013b]. In the literature, MRKH syndrome is also referred as Müllerian agenesis, Müllerian aplasia, or congenital absence of the uterus and vagina and is generally divided in two categories. Type 1 MRKH (complete or typical form) features complete absence of uterus and vagina without any associated malformations. Completely asymptomatic individuals with uni- or bilateral vestigial remnants of mullerian tissue that do not contain any endometrial tissue are still categorized as Type 1. Type 2 MRKH (incomplete or atypical form) has either partial absence of uterus and vagina and/or additional associated malformations (frequently renal and spinal malformations; less frequently cardiac, ocular, auricular anomalies, and inguinal herniae [Rall et al., 2015b]). Individuals with small remnants of mullerian tissue, which are symptomatic because of the presence of some functioning endometrial tissue, are categorized as Type 2. MURCS (Müllerian, renal, and cervicothoracic somite) association represents one specific subtype of type 2 MRKH [Dietrich et al., 2014].

TABLE I. Comparison of the Main Classification Systems for Uterine Malformations

	ESHRE/ESGE classification system Grimbizis et al. [2013]	Updated classification for female genital tract malformation Acién and Acién [2011]	Vagina, cervix, uterus, adnexae, and associated malformations system (VACUAM system) Oppelt et al. [2005]	AFS/ASRM classification system American Fertility Society [1988]
Main classes for uterine malformations	U0, normal uterus	(1) Unilateral genito-urinary agenesis or hypoplasia (with or without contralateral Müllerian agenesis)	(U)0 Normal	Class I, aplasia, hypoplasia
	U1, dysmorphic uterus ((a) T-shaped, (b) infantilis, (c) others)	(2) Uterine duplicity with a blind hemivagina (or atresia) and ipsilateral renal agenesis	(U)1 Arcuate (1a)-septate (1b–c)	Class II, unicornuate
	U2, septate uterus ((a) partial, (b) complete)	(3) Isolated or common uterine or utero-vaginal anomalies	(U)2 Bicornate	Class III, didelphys
	U3, bicorporeal uterus ((a) partial, (b) complete, (c) bicorporeal septate)	(3A) Any of the ASRM-classified Müllerian ducts anomalies based on ASRM classification	(U)3 Hypoplastic uterus	Class IV, bicornuate
	U4, hemi-uterus ((a) with or (b) without a rudimentary cavity)	(3B) Müllerian tubercle anomalies	(U)4 Rudimentary or aplastic unilaterally (4a), bilaterally (4b)	Class V, Septate
	U5, aplastic uterus ((a) with or (b) without rudimentary cavity)	(3C) Both Müllerian ducts and tubercle anomalies	(U)# Unknown	Class VI, arcuate
	U6, for still remaining unclassified cases	(4) Accessory uterine masses and other gubernaculum dysfunctions	+ Includes an independent classification for vagina (V), cervix (C),and adnexa (A)	Class VII, DES-related anomaly
	+ Include an independent classification system for cervical and vaginal anomalies	(5) Anomalies of the urogenital sinus		
		(6) Malformative combinations		
Renal anomalies	Reported in the addendum "Associated non-Müllerian anomalies"	Included in the classification system	Reported by the letter (R) if present	Not included in the classification system
Advantages	Systematic and precise reflection of the entire malformation	Accounts for frequently associated anomalies	Systematic and precise reflection of the entire malformation Takes into account associated malformations	Simple, user-friendly, widely used
Limitations	Recently introduced; clinical utility will be determined over time	Unwieldy for use in clinical practice with difficulties in assignment to specific classes	More complex to use clinically Does not suggest the aethiopathogenicity	Lack of classification for complex malformations resulting from a dependence of classification systems for uterus, cervix, and vagina

Based on Grimbizis et al. [2013]; Acién and Acién [2011]; Oppelt et al. [2005]; The American Fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, Müllerian anomalies, and intrauterine adhesions [1988].

Other terms that have been used in the literature for specific associations are the terms Herlyn–Werner–Wunderlich syndrome and OHVIRA (obstructed hemivagina, and ipsilateral renal agenesis) syndrome which both describe a complete bicorporeal uterus with a double cervix (didelphys uterus) and a longitudinal obstructing vaginal septum associated with renal agenesis ipsilateral to the obstructed hemivagina [Smith and Laufer, 2007]. Finally, the ESHRE/ESGE classification includes two other classes of uterine anomalies. Dysmorphic uteri (class U1 in ESHRE/ESGE classification) such as infantilis uterus or T-shaped uterus have been reported after prenatal drug exposure (discussed at the end of this review) and when untreated, are frequently associated with unfavorable fertility and pregnancy outcome [Angelis and Caserta, 2015]. Infantilis uteri may be in some cases secondary to ovarian dysgenesis (e.g., as in Turner syndrome) and have also been reported in other syndromic conditions (Table IV). The category of unclassified uterine malformation (class U6 in ESHRE/ESGE classification) covers combined pathologies or extremely rare malformations such as a duplication defect, which can be a feature of the caudal duplication syndrome (see Table IV for description).

Cohorts of women with uterine anomalies that have been studied for prevalence, recurrence, or genetic etiologies of congenital uterine anomalies have usually had admixed types of uterine anomalies (mainly U2, U3, U4, and U5, as described above); or studies have focused on Mayer–Rokitansky–Kuster–Hauser syndrome (U5C4V4 according to the ESHRE/ESGE classification). The extra-uterine phenotypes are also usually variable within most study cohorts, including some individuals with isolated uterine anomalies, others with a complex, non-isolated phenotype (typically with involvement of kidneys or vertebrae), and sometimes those with uterine malformations in the context of a recognizable genetic syndrome. In this review, we use the term syndromic uterine malformation in reference to Mendelian genetic syndromes. Therefore, MRKH syndrome and OHVIRA syndrome are in fact considered part of the isolated and non-isolated uterine malformation category (when they were not reported with other features in addition to kidney or vertebral anomalies). The reason, as will be made clear below, is that the causation for these malformation complexes appears to be multifactorial/polygenic, not Mendelian.

In this review, the ESHRE/ESGE classification will be used when referring to uterine malformation except when a specific association is described (e.g., MRKH syndrome) or when the equivalent in the new classification cannot be established from the description reported (e.g., "hypoplastic uterus" could refer either to a dysmorphic uterus or an aplastic uterus with rudimentary cavities).

PREVALENCE

The prevalence of uterine malformations has been estimated to be as high as 5.5–9.8% in a general female population [Chan et al., 2011; Dreisler and Stampe Sørensen, 2014]. These estimates include the relatively high frequency of arcuate-shaped uteri (3.9–6.8%). Though this configuration is considered by some as a normal variant rather than a true malformation, it does reflect a minor degree of a septation defect [Dietrich et al., 2014]. The meta-analysis of Chan et al. [2011], involving a total of 5,163 women, reported a prevalence of 2.3% for partial and complete septate anomalies (canalization defects), a prevalence of 0.7% for bicorporeal uteri (0.4% for bicornuate uteri and 0.3% for didelphys uteri), and 0.1% for hemi-uterus (unicornuate uteri). MRKH syndrome is rare, with an incidence of 1 in 4,500–5,000 newborn females [Aittomäki et al., 2001].

RECURRENCE RISK FOR UTERINE MALFORMATIONS

The etiology of most uterine malformations is generally believed to be multifactorial. An estimated recurrence risk of 1–5% for first-degree relatives is usually quoted to families, and the few existing studies (Table II) have identified a degree of risk for siblings that is similar to most other multifactorial disorders. Numerous reports of discordant phenotypes in monozygotic twins (e.g., MRKH syndrome vs. normal uterus) argue against strong heritability and suggest post-zygotic somatic mutation or non-genetic mechanisms such as epigenetic or microenvironmental factors underlie the occurrence of congenital uterine malformations [Halbert and Christakos, 1970; Lischke et al., 1973; Heidenreich et al., 1977; Regenstein and Berkeley, 1991; Steinkampf et al., 2003; Duru and Laufer, 2009; Milsom et al., 2015; Rall et al., 2015a,b]. However, whereas these mechanisms may explain sporadic cases, reports of familial cases do support the existence of a predisposing genetic background that may be stronger in some families compared to others. According to Hammoud et al. [2008], 10% of causality might be attributed to familial affiliation, a term they use to encompass shared genetic and environmental factors in families. They reported a 12-fold increased risk for first-degree relatives of patients affected by MDA relative to background incidence, and detectable increased risk in second and third-degree relatives, although the latter was not statistically significant [Hammoud et al., 2008]. Moreover, it is likely that the role of inherited factors and the true rate of familial recurrence have been underestimated in a majority of existing studies. Uterine ultrasounds were usually not performed, or not performed in extended family members and asymptomatic or subtle anomalies were unlikely to be ascertained by report alone. On the one hand, it may be clinically appropriate to ascertain recurrence only for those anomalies that are functionally/clinically obvious, as this is what patients may be most concerned about; but on the other hand, it is necessary to consider subtle anomalies as part of the spectrum occurring within families in order to truly understand the genetic contribution to causation. In most families, recurrences tend to recapitulate the same type of uterine malformation, but recurrences with variable types of uterine maldevelopment also have been reported in several families. In some families, these malformations even appear to result from defects at different developmental stages (formation or fusion/septation defects) [Hay, 1961; Buchta et al., 1973; Griffin et al., 1976; Verp et al., 1983; Heidenreich, 1988; Pavanello et al., 1988; Aughton, 1993; Hammoud et al., 2008; Lalatta et al., 2015] suggesting that similar genetic background could still predispose to different types of

TABLE II. Studies Evaluating Recurrence of Urogenital or Uterine Malformations in Families of Patients With Uterine Malformations

Study	Number of probands	Anomalies	Relatives studied	% individuals with a family history of urogenital malformation[a]	Recurrence risk of urogenital malformation for siblings/relatives of a woman with MRKH/congenital uterine anomalies	Method
Rall et al. [2015b]	246[b]	Typical MRKH (n = 184) Atypical MRKH (n = 143) MURCS syndrome (n = 19)	First-degree relatives	39/246 (15%) (U/R)[c]	NA	Questionnaire
Hammoud et al. [2008]	1,376[d]	High probability of Müllerian anomalies (ascertainment limited to billing and ICD codes)	First- and second-degree relatives	19/1376 (1.4%) (U)	Relative risk (U): First-degree relatives: 11.6 ($P < 0.001$) Parent/children: 8.78 ($P < 0.001$) Siblings: 12.98 ($P < 0.001$)	Kinship analysis from a large population database
	1,397		Family clustering (≥5 affected descent)	256/1,397 (18%) (U)	First cousins: 1.44 ($P = 0.29$) Second cousins: 1.30 ($P = 0.11$)	
Wottgen et al. [2008]	59[b]	Typical MRKH (n = 45) Atypical MRKH (n = 12) MURCS (n = 16)	Siblings	1/59[e] (1.7%) (U/R)	Siblings: 0.97% (1/103 siblings) (U/R)	Questionnaire
Petrozza et al. [1997]	58[f]	MRKH (surrogate pregnancies)	Offspring	NA	Offspring: 0% (0 (U/R)/17 females and 0 (R)/17 males)	Questionnaire
Pavanello et al. [1988]	20 (11)[g]	MRKH	First-degree relatives	4/11 (36.4%) (U/R)	Siblings: 3.2% (±1.8%) e (U/R)	US examination
Elias et al. [1984]	24	Bicorporeal uterus (n = 9) Hemi-uterus (n = 2) Septate uterus (n = 11) Arcuate uterus (n = 2)	First- and second-degree relatives	1/24 (4.2%) (U)	Female siblings: 2.7% (1/37) (female siblings/U)	Interviews
Carson et al. [1983]	23	Aplastic uterus (n = 22) Other uterine malformation (n = 1)	First- and second-degree relatives	0/23 (0%) (U)	Female siblings: 0% (0/30 post-pubertal sisters) (U)	Interviews
van Lingen et al. [1998][h]	34	MRKH	Siblings	1/34 (2.9%) (U)	Female siblings: 2.9% (1/34 post-pubertal sisters) (U)	Observations in context of molecular studies

MRKH, Mayer–Rokitansky–Kuster–Hauser syndrome (U5C4V4 in the ESHRE/ESGE classification); MURCS, Müllerian, renal, and cervicothoracic somite syndrome; NA, data non available.
[a]Evaluation of recurrence for uterine (U) or renal abnormalities (R).
[b]Number of individuals out of the total number who had siblings *and* completed the questions with respect to siblings.
[c]This number also includes individuals with a family history of skeletal malformations or other malformations.
[d]1397 − 21 = 1376 (subtraction of 21 individuals in order to consider only one proband per family when there are multiple affected family members).
[e]Occurrence of unilateral testis in one sibling was not included because a primary gonadal malformation indicated distinct developmental pathways, and was probably unrelated to MRKH in the affected proband.
[f]Represents the total number of probands in the study. However, the number of women who actually had offspring is not available.
[g]Twenty probands were included but only 11 sibships were investigated by ultrasound. The number of siblings for these 11 families was not available. The recurrence risk for siblings to be affected with any type of Müllerian or renal defect is probably underestimated because it refers to the total 20 sibships.
[h]Referred to in Simpson [1999].

uterine malformation with variable penetrance and expressivity. Consequently, it may not be possible to predict the severity of a recurrence based on the phenotype of a proband. Finally, reports of a large number of families in the literature with recurrence of Müllerian and kidney anomalies or Müllerian and axial skeletal anomalies suggest that radiological investigation of these systems should be considered in relatives before calculating genetic attribution in research [Griffin et al., 1976; Schimke and King, 1980; Verp et al., 1983; McPherson et al., 1987; Pavanello et al., 1988; Bingham et al., 2002; Morcel et al., 2011; Herlin et al., 2014]. Estimates of recurrence for clinical purposes ideally should also include risk estimates for renal anomalies. In families with associated renal and uterine malformations, hereditary urogenital adysplasia [Schimke and King, 1980] may be the most appropriate diagnosis, which features variable expressivity and decreased penetrance.

GENETIC FACTORS ASSOCIATED WITH HUMAN UTERINE MALFORMATIONS

To date, the primary approach to identify genes potentially involved in human uterine malformations has started with hypotheses regarding candidate genes based on animal models, followed by targeted assays (usually Sanger sequencing) in affected and control populations, in an effort to prove or disprove an association. Additional proposed candidates have been based on known gene–gene interaction networks. Monogenic multiple malformation syndromes that can involve the uterus have also been a source for potential candidate causes for isolated uterine malformations. Finally, recurrences of several copy number variants found in up to 14% of affected individuals [Nik-Zainal et al., 2011] have suggested interesting candidate genes. In this section, Figure 1, and Table III, we review the genetic factors that have been associated with human uterine malformations. Candidate genes that have been interrogated by targeted sequencing in cohorts of individuals with uterine malformations are reported in Table III. We have provided interpretation of the pathogenicity of these variants in accordance with ACMG Variant Interpretation Guidelines [Richards et al., 2015]. Therefore, we have excluded many candidate variants reported in the literature because it is now apparent they are far too prevalent in large databases such as ExAc to be independently pathogenic. Some of these candidate genes are also responsible for syndromic conditions in which uterine malformations are a frequent feature, sometimes being the dominant finding leading to the diagnosis. In order to describe all variants that have been associated with a predominant uterine phenotype for the gene, individuals with those syndromic conditions are also included in Table III. Variants identified in individuals with an isolated uterine phenotype are specified in this table.

Figure 1 summarizes all of the genes associated at least once with an isolated uterine malformation in humans. Lines of evidence for causality of these genes come from epidemiological and experimental data, including murine models, and are reviewed in this section. More extensive reviews of genes involved in uterine phenotypes in mice-only are available elsewhere [Massé et al., 2009; Mullen and Behringer, 2014].

HNF1B and Hereditary Urogenital Dysplasia

HNF1B (hepatocyte nuclear factor-1B; also known as *TCF2*) was the first gene with pathogenic variants found in individuals with uterine malformations. Lindner et al. [1999] reported a family with renal cysts and diabetes syndrome (MODY type 5) in which two sisters presented with an aplastic uterus with vaginal aplasia (MRKH), kidney disease, and diabetes. Since then, 19 unrelated individuals with syndromic and variable uterine malformations have been associated with heterozygous *HNF1B* mutations (Table III). Notably, all of these individuals, except one, have presented additionally with renal disease and/or diabetes. Moreover, 10 patients with a 17q12 deletion, including *HNF1B* and other contiguous genes, have been reported with both typical and atypical MRKH anomaly [Cheroki et al., 2008; Bernardini et al., 2009; Ledig et al., 2011; Nik-Zainal et al., 2011; Sandbacka et al., 2013]. *HNF1B* is a POU homeodomain-containing transcription factor. Gene inactivation in mouse embryos is lethal before the gastrulation stage [Lokmane et al., 2010], and Hnf1β has been shown to be strongly expressed in Wolffian and Müllerian ducts during development [Coffinier et al., 1999]. Hnf1β positively regulates Lim1, Pax2, and Wnt9b expression in mice [Lokmane et al., 2010]. These three genes are associated with partial or complete absence of the FGT in knockout mice [Massé et al., 2009]. Expression of Lim1, Wnt9b, and Pax8 has been shown to be altered in the primary stage of Müllerian ducts formation in Hnf1β mutants [Lokmane et al., 2010]. In humans, lack of expression or down-regulation of these genes by *HNF1B* haploinsufficiency might lead to abnormal uterine phenotypes. To date, *HNF1B* is the only gene in which mutations have been found in families with recurrence of uterine plus renal malformations, a condition named hereditary urogenital adysplasia by Schimke and King [1980]. Oram et al. [2010] noted a prevalence of *HNF1B* mutation in 18% of women with both uterine and kidney abnormalities [Oram et al., 2010]. However, *HNF1B* sequencing of a series of patients with isolated uterine anomalies did not uncover any mutations [Bingham et al., 2002; Bernardini et al., 2009; Oram et al., 2010; Ledig et al., 2011]. Overall, *HNF1B* mutation or deletion seems to be rarely associated with isolated uterine anomalies in the absence of renal abnormalities (in either the affected individuals or their relatives), but testing should be considered in individuals with a family history suggestive of hereditary urogenital adysplasia. The pattern of inheritance is autosomal dominant with incomplete penetrance and variable expressivity. Incomplete penetrance and heterogeneity of the uterine phenotype could possibly be explained by contributions from other genetic variants (e.g., mutations or functional polymorphisms) or epigenetic factors, for example, those that result in temporal variation in *HNF1B* expression, or its close interactors [Clissold et al., 2015].

HOX Genes

During embryonic development, transcription factors encoded by the conserved homeobox (HOX) genes act to determine the anteroposterior patterning of several organs. HOX genes are clustered on four chromosomes. The most 5′ genes of clusters A and D, Hoxa9–13 and Hoxd9–13, are expressed in developing

TABLE III. Candidate Genes Interrogated in Cohorts With Uterine Malformations

Gene	Individuals with UM tested for the gene (number of positive/total number of individuals tested)	Cohort's phenotype/ethnicity	Uterine phenotypes in individuals tested positive/number reported with isolated UM	Variant (number of unrelated individuals and genotype)	Likelihood of pathogenic significance
AMH	Zenteno et al. [2004] (0/15)	MRKH/Mex	–	–	–
AMHR	Zenteno et al. [2004] (0/15)	MRKH/Mex	–	–	–
CFTR	Timmreck et al. [2003a] (0/25)	MRKH/NR	–	–	–
CTNNB1 *GSK-3β phosphorylation sites on exon 3*	Drummond et al. [2008] (0/12)	MRKH/NR	–	–	–
DLGH1	Ravel et al. [2012] (0/12)	MRKH/E	–	–	–
EMX2	Liu et al. [2015] (1/517)	UM/C	U3bC2/1	p. (Glu14*) (1 Hz)	Uncertain significance
GALT *exon 10*	Zenteno et al. [2004] (0/15)	MRKH/Mex	–	–	–
HNF1B	Alvelos et al. [2015] (CR)	–	U3/0	p. (Asn38 fs) (1 Hz)	Pathogenic
	Ledig et al. [2011] (0/56)	MRKH/NR	–	–	–
	Oram et al. [2010] (9/108)[a]	UM/NR	U3, U4, U5/0	p. (Met1_Trp557del) (4 Hz)[b]	Pathogenic
				p. (Ala373fs) (1 Hz)	Pathogenic
				p. (Val61Gly) (1 Hz)	Uncertain significance
				p. (Cys273Ser) (1 Hz)	Likely pathogenic
				c.544 + 3_ 544 + 6del (1 Hz)	Likely pathogenic
				p. (Ser151Pro)(1 Hz)	Likely pathogenic
	Heidet et al. [2010] (CR)	–	U2, U3, U3bC2, U5/0	p. (Met1_Trp557del) (4 Hz)[b]	Pathogenic
				p. (Glu78*) (1 Hz)	Pathogenic
				p. (Ser379*) (1 Hz)	Pathogenic
	Bernardini et al. [2009] (0/20)	MRKH/NR	–	–	–
	Shihara et al. [2004] (CR)	–	U3/0	p. (Leu95fs) (1 Hz)	Pathogenic
	Bingham et al. [2002] (2/9)[a]	UM/NR	U3; U3bC2/1[c]	p. (Gln243 fs) (1 Hz)	Pathogenic
				p. (Ser151Pro) (1 Hz)	Likely pathogenic
	Iwasaki et al. [2001] (CR)	–	U3/0	c.544 + 1G>A (1 Hz)	Pathogenic
	Lindner et al. [1999] (CR)	–	U5/0	p. (Arg137_Lys161del) (1 Hz)	Likely pathogenic
HOXA7	Chen et al. [2014b] (0/192)	UM/C	–	–	–
	Burel et al. [2006] (0/6)	MRKH/NR	–	–	–
HOXA9	Chen et al. [2014b] (1/192)	UM/C	U3bC2/1	c.*73C>G	Uncertain significance
	Burel et al. [2006] (0/6)	MRKH/NR	–	–	–
HOXA10	Ekici et al. [2013] (4/27)	UM/NR	U2, U3/3	p. (Gly51Asp) (2 Hz)	Uncertain significance
				p. (Arg371 fs) (1 Hz)	Uncertain significance
				c.-224C>A (1 Hz)	Uncertain significance
				c.147G>A (1 Hm)[d]	Uncertain significance
	Cheng et al. [2011] (1/109)	UM/C	U3bC2/1	p. (Tyr57Cys) (1 Hz)	Uncertain significance
	Liatsikos et al. [2010] (0/30)	UM/G	–	–	–
	Lalwani et al. [2008] (0/26)	MRKH/NR	–	–	–

(*Continued*)

TABLE III. (*Continued*)

Gene	Individuals with UM tested for the gene (number of positive/total number of individuals tested)	Cohort's phenotype/ethnicity	Uterine phenotypes in individuals tested positive/number reported with isolated UM	Variant (number of unrelated individuals and genotype)	Likelihood of pathogenic significance
	Burel et al. [2006] (0/6)	MRKH/NR	–	–	–
HOXA11	Chen et al. [2014a, 11] (4/192)	UM/C	U2, NR/4	c.774G>A (1 Hz)	Uncertain significance
				c.942 + 9C>T (3 Hz)	Uncertain significance
	Liatsikos et al. [2010] (1/30)	UM/G	U2/0	p. (Pro38Arg) (1 Hz)	Uncertain significance
	Burel et al. [2006] (0/6)	MRKH/NR	–	–	–
HOXA13	Imagawa et al. [2014] (CR)	–	U3/0	p. (Ile368Phe) (1 Hz)	Pathogenic
	Ekici et al. [2013] (0/27)	UM/NR	–	–	–
	Parker et al. [2011] (CR)	–	U3bC2/0	p. (Arg326Gly) (1 Hz)	Pathogenic
	Jorgensen et al. [2010] (CR; 0/17)	UM[e]/NR	U2/0	p. (133Ala$_{10}$)[f] (1 Hz)	Pathogenic
	Utsch et al. [2007] (CR)	–	U3/0	p. (133Ala$_{14}$)[f] (1 Hz)	Pathogenic
	Burel et al. [2006] (0/6)	MRKH/NR	–	–	–
	Innis et al. [2004] (CR)	–	U2, U3bC2/0	p. (133Ala$_{11}$) (1 Hz)	Pathogenic
				p. (133Ala$_{12}$)[f] (1 Hz)	Pathogenic
	Goodman et al. [2000] (CR)	–	U2, U3, U3bC2/0	p. (Gln365*) (1 Hz)	Pathogenic
				p. (133Ala$_{8}$)[f] (1 Hz)	Pathogenic
	Mortlock and Innis [1997] (CR)	–	U3bC2/0	p. (Trp369*) (1 Hz)	Pathogenic
LAMC1	Ravel et al. [2012] (0/12)	MRKH/E	–	–	–
LHX1	Sandbacka et al. [2013] (5/112)	MRKH/F	U5/5	p. (Cys4Ser) (1 Hz)	Uncertain significance
				p. (Pro312His) (3 Hz)[g]	Uncertain significance
				p. (Pro332Arg) (1 Hz)	Uncertain significance
	Xia et al. [2012] (1/96)	UM/C	U3bC2/0	p. (Pro357_Ser360del) (1 Hz)	Uncertain significance
	Ledig et al. [2012] (1/62)	MRKH/NR	U5/0	p. (Arg9 fs)	Uncertain significance
	Ledig et al. [2011] (1/56)	MRKH/NR	U5/1	p. (Arg264Gly)	Uncertain significance
	Bernardini et al. [2009] (0/20)	MRKH/NR	–	–	–
PAX2	Wang et al. [2012] (1/192)	UM/C	U3bC2/1	c.320G>A[h]	–
PBX1	Ma et al. [2011, 1] (0/192)	UM/C	–	–	–
	Burel et al. [2006] (0/6)	MRKH/NR	–	–	–
RBM8A	Tewes et al. [2015] (3/167)	UM/NR	U5/2	p. (Tyr124Phe) (1 Hz)	Uncertain significance
				c.*25C>G (2 Hz)	Uncertain significance
RARG	Cheroki et al. [2006] (0/25)	MRKH/NR	–	–	–
RXRA	Cheroki et al. [2006] (0/25)	MRKH/NR	–	–	–
TBX6	Tewes et al. [2015] (6/167)	UM/NR	U5, U2, U3/4	p. (Pro339Thr) (1 Hz)	Uncertain significance
				p. (Gly162Ser) (5 Hz)[i]	Probable functional polymorphism
	Sandbacka et al. [2013] (17/112)	MRKH/F	U5/17	p. (Gly162Ser) (12 Hz/2Hm)	Probable functional polymorphism
				p. (Arg272Gln) (9 Hz/2Hm)[i]	Probable functional polymorphism
				c.622-2A>T (2 Hz)	Uncertain significance
WNT4	Waschk et al. [2015] (0/14)	UM/E	–	–	–
	Chang et al. [2012] (1/189)	UM/C	U4/1	c.1091G>A (1 Hz)[h]	–
	Philibert et al. [2011] (1/4)	MRKH/NR	U5/0	p. (Ala233Thr) (1 Hz)	Uncertain significance

(*Continued*)

TABLE III. (*Continued*)

Gene	Individuals with UM tested for the gene (number of positive/total number of individuals tested)	Cohort's phenotype/ethnicity	Uterine phenotypes in individuals tested positive/number reported with isolated UM	Variant (number of unrelated individuals and genotype)	Likelihood of pathogenic significance
	Ravel et al. [2009] (1/11)	MRKH/E	U5/0	c.483C>T (1 Hz)[j]	Uncertain significance
	Philibert et al. [2008] (1/25)	MRKH/NR	U5/0	p. (Leu12Pro) (1 Hz)	Likely pathogenic
	Biason-Lauber et al. [2007] (1/6)	UM/NR	U5/0	p. (Arg83Cys) (1 Hz)	Likely pathogenic
	Cheroki et al. [2006] (1/25)	MRKH/NR	NR	c.1026C>T (1 Hz)[h]	–
	Clément-Ziza et al. [2005] (0/19)	MRKH/NR	–	–	–
	Biason-Lauber et al. [2004] (CR)	–	U5/0	p. (Glu216Gly) (1 Hz)	Likely pathogenic
WNT5A	Wu et al. [2013] (1/189)	UM/C	U4/1	c.-528G>A (1 Hz)	Uncertain significance
	Ravel et al. [2009] (0/11)	MRKH/E	–	–	–
WNT7A	Al-Qattan et al. [2013] (CR)	–	U5/0	p. (Arg292Cys) (1 Hm)[k]	Pathogenic
	Dang et al. [2012] (1/191)	UM/C	U3/1	p. (Gly114Gly) (1 Hz)	Uncertain significance
	Eyaid et al. [2011] (CR)	–	U5/0	p. (Gly204Ser) (1 Hm)[k]	Pathogenic
	Ravel et al. [2009] (1/11)	MRKH/E	U5/0	c.861G>A (1 Hz)[j]	Uncertain significance
	Timmreck et al. [2003b] (0/40)	UM/NR	–	–	–
WNT9B	Waschk et al. [2015] (6/226)	UM/E	U5, U3/6	p. (Gln158Glu) (1 Hz)	Uncertain significance
				p. (Arg222His)[l] (1 Hz)	Uncertain significance
				p. (Arg241His) (1 Hz)	Uncertain significance
				p. (Arg307Trp)[l] (1 Hz)	Uncertain significance
				p. (Arg325His) (1 Hz)	Uncertain significance
				p. (Cys343*) (1 Hz)	Uncertain significance
	Wang et al. [2014] (1/42)	MRKH/C	U5/1	p. (Ala10Ser) (1 Hz)[m]	Uncertain significance
				c.*158C>T (1 Hz)[m]	Uncertain significance
	Tang et al. [2014] (0/191)	UM/C	–	–	–
	Ravel et al. [2009] (0/11)	MRKH/E	–	–	–

Uterine phenotypes are reported following the ESHRE/ESGE classification (U5 = aplastic uterus, U4 = hemi-uterus, U3 = bicorporeal uterus, U3bC2 = complete bicorporeal uterus with a double cervix [formerly didelphys uterus], U2 = septate uterus); CR, case report; UM, uterine malformations (i.e., all types of uterine malformations, including MRKH); Ht, heterozygous variant; Hm, homozygous variant; ethnic groups: E, European; C, Chinese; F, Finnish; G, Greek; Mex, Mexican; NR, not reported or diverse ethnicity reported.

[a]One positive individual is reported in two cohorts [Bingham et al., 2002; Oram et al., 2010].

[b]In these cases of whole gene deletion, the exact size of the deletion was usually not specified and could represent contiguous gene deletions in the 17q12 region.

[c]One patient presented with isolated uterine malformation. However, her relatives presented with symptoms of RCAD.

[d]Associated with the heterozygous variant p.Gly51Asp in one individual [Ekici et al., 2013].

[e]Biopsy samples from 17 individuals with idiopathic uterine or uterovaginal septa [Jorgensen et al., 2010].

[f]Expansion by 8–14 residues of the third polyalanine tract including usually 18 alanines.

[g]Associated with *TBX6* variants in two individuals [Sandbacka et al., 2013].

[h]Location of the variant was unclear based on the publication [Wang et al. 2012].

[i]Two individuals, who were homozygotes for both rare polymorphisms, presented, moreover, a *LHX1* variant and an 11q13.4 deletion, respectively. One individual was found with a 22q11.21 deletion and a heterozygous rare polymorphism. One individual was heterozygous for both polymorphisms and presented with a *LHX1* variant [Sandbacka et al., 2013; Tewes et al., 2015].

[j]This individual was identified with a variant in *WNT4* and a variant in *WNT7A* [Ravel et al., 2009].

[k]Autosomal recessive syndromic cases.

[l]The missense variant p. (Arg222His) was associated with a deletion in the chromosomal region 17q12 including *LHX1* in one individual. The missense mutation p. (Arg307Trp) was associated with a missense variant in TBX6 (p. (Arg272Gln)) [Waschk et al., 2015].

[m]Both variants were identified in one individual (it is unclear whether they were *in cis* or *in trans*).

Müllerian ducts in mice [Massé et al., 2009]. Following an anteroposterior axis of expression, Hoxa-9 is most expressed in the fallopian tubes, Hoxa-10 in the uterus, Hoxa-11 in the uterus and uterine cervix, and Hoxa-13 in the upper vagina [Taylor et al., 1997]. Hoxd9–11 are expressed in the uterus during development with a similar pattern of expression [Raines et al., 2013] and Hoxd13 in the caudal portion of the Müllerian ducts [Warot et al., 1997]. Functional redundancy is a key feature of the Hox gene family. In the murine uterus, redundancy in function as well as synergistic effects have been observed for flanking Hox genes (Hoxa9–11) and for paralogous genes from different clusters (Hoxa9–11 and Hoxd9–11) [Raines et al., 2013]. Partial and complete anteriorization of the uterus—meaning a homeotic transformation to a morphology and histology resembling oviducts—can be observed in Hoxa10 and Hoxa11 knockout mice, respectively [Benson et al., 1996; Gendron et al., 1997]. A more severe phenotype was noted in mice with flanking null mutations simultaneously introduced for Hoxa9–11, which was further worsened when accompanied by flanking heterozygous mutations introduced for Hoxd9–11 [Raines et al., 2013]. Flanking homozygous loss of Hoxd9–11 on its own, however, is not sufficient to cause a uterine phenotype. Hoxa13 knockout mice lack the caudal portion of the Müllerian ducts [Warot et al., 1997]. Hoxd13$^{-/-}$ female mice were shown to be fertile (internal urogenital organs not described) [Dollé et al., 1993], but compound Hoxa13$^{+/-}$, Hoxd13$^{-/-}$ mice were sterile with uterine and/or vaginal abnormalities (partial agenesis of uterus and vagina, septated vagina), renal and rectal abnormalities [Warot et al., 1997]. Hoxa9–13 and Hoxd9–13 are also particularly important in vertebrate limb development [Du and Taylor, 2004] and several Hox genes are expressed in the developing kidney, including Hoxa9–11 and Hoxd9–11 [Patterson and Potter, 2004].

Rare variants of uncertain significance have been identified in *HOXA10* and *HOXA11* in 10 individuals with sporadic uterine malformations. Further epidemiological or biological evidence is needed before concluding any of these variants affect uterine development. Pathogenic variants in *HOXA13* are associated with hand–foot–genital (HFG) syndrome. HFG syndrome is characterized by limb malformations and urogenital defects, which can include defects of Müllerian fusion and septal absorption to a variable degree. Missense variants result in a more severe phenotype compared to a presumed loss-of-function mechanism with frameshift variants, gene deletion, and polyalanine expansions [Imagawa et al., 2014], and hence a possible dominant negative or gain of function effect has been suspected. Two variants in *HOXA13* with contradictory predictions by in silico programs have been reported in two individuals with MRKH. These variants were absent in a small cohort of 53 controls [Ekici et al., 2013] and are reported with minor allele frequencies of 0.08 (rs34185333) and 0.04 (rs35042646) in the European non-Finnish populations of ExAc.

EMX2

EMX2 encodes a homeobox-containing transcription factor expressed in urogenital epithelial tissues, the dorsal telencelphalon, and the olfactory epithelium during development. Female knockout mice lack the genital tract, kidneys, and gonads [Miyamoto et al., 1997] and also present with brain malformations. *EMX2* expression has been shown to be repressed by *HOXA10* [Taylor, 2015] and one of its target genes might be *TP63* [Liu et al., 2015]. Liu et al. [2015] demonstrated expression of a transcript in human adult uterus (NM_004098.3; NP_004089.1) and sequenced the gene in 517 women with uterine malformations (fusion or septation defects) and 563 controls. One heterozygous nonsense variant resulting in a truncated protein lacking the homeodomain was identified in an individual with an isolated complete bicorporeal uterus with a double cervix (didelphys uterus). A dominant negative effect for this variant was suspected following functional studies of transfected human embryonic kidney cells [Liu et al., 2015]. Remnants of Müllerian ducts were found in a male with a 10q26 deletion including *EMX2* [Piard et al., 2014].

LHX1

In the largest MRKH case–control study to date, the rare pathogenic deletion in the region 17q12 was found in several MRKH-affected individuals with a statistically significant difference compared to controls [Cooper et al., 2011, Nik-Zainal et al., 2011]. Besides *HNF1B* (discussed above), the region also includes *LHX1*, another interesting gene for Müllerian anomalies. Involvement of *LHX1* in uterine malformation is supported by studies of animal models. *LHX1* encodes a LIM-homeodomain transcription factor and its expression is likely essential in the Müllerian duct epithelial progenitor cells for ductal elongation and uterine endometrium differentiation. Loss of expression leads to shortened oviducts, uterine aplasia, and infertility in Müllerian duct-specific Lhx1 conditional knockout mice [Huang et al., 2014]. Downstream target genes are currently unknown which limits functional analysis of candidate variants. To date, from five series of individuals with MRKH or Müllerian duct anomalies (Table III), eight variants of uncertain significance have been identified in *LHX1*. Should further evidence accumulate to support pathogenicity of these variants, then *LHX1* variants might be involved in 2% of individuals with uterine malformations.

PAX2

Loss of the transcription factor gene *pax2* (paired box gene 2) in homozygous knockout mice causes agenesis of kidneys, ureters, and genital tracts [Torres et al., 1995]. Pax-2 is expressed in both Wolffian and Müllerian ducts [Torres et al., 1995]. However, it has not been determined yet if absence of female reproductive tracts result from defective action of *PAX2* in the Müllerian ducts or if this disturbance is secondary to abnormal wolfian duct development. Among 192 individuals with various uterine malformations, a rare synonymous variant not found in controls was reported in one individual with a complete bicorporeal uterus and absence of fallopian tubes [Wang et al., 2012]. In humans, *PAX2* mutations are associated with papillorenal syndrome and isolated renal hypoplasia [Vivante et al., 2014].

RBM8A

The region 1q21.1, which includes the gene *RBM8A*, is another locus for which recurrent copy number variants have been identified in individuals with MRKH. When deleted in the presence of

certain low-frequency noncoding polymorphisms in *RBM8A*, TAR (thrombocytopenia-absent radius) syndrome results. Five individuals with TAR syndrome have also had MRKH, one of whom was documented as having a 1q21.1 deletion [Behera et al., 2005; Childs and Hall, 2005; Griesinger et al., 2005; Ahmad and Pope, 2008; Ledig et al., 2011]. Recently, Tewes et al. [2015] analyzed the gene *RBM8A* in 167 individuals with MRKH or other Müllerian duct anomalies. They identified the TAR-associated variants (rs139428292) and (rs201779890) with a MAF of 3.6% and 0.3%, respectively. These variants are reported with similar frequencies in ExAC considering the European non-Finnish population (3.4% and 0.9%, respectively) [Tewes et al., 2015; Exome Aggregation Consortium]. Moreover, one rare missense and one rare intronic variant (rs201860373) were identified in one individual with MRKH type 2 and two individuals with MRKH type 1, respectively. To date, there is no strong evidence implicating the gene *RBM8A* in the pathogenesis of uterine malformation, and although *RBM8A* could be a candidate gene, wider genetic analyses of affected populations would be necessary to determine the causality of variants in this gene.

TBX6

Being involved in mesodermal development, *TBX6* appeared to be region 16p11.2's most likely susceptibility gene for uterine malformations. Deletions in 16p11.2 (600 kb) have been identified in 2–6% of individuals in small series of patients with MRKH [Nik-Zainal et al., 2011; Sandbacka et al., 2013], which is a much higher frequency than the 0.03–0.04% observed in the general population [Cooper et al., 2011; Tucker et al., 2013]. Enriched prevalence of this CNV is also found in cohorts ascertained for intellectual disability/multiple congenital anomalies (0.33%), autism spectrum disorder (0.7–1%), morbid obesity (0.7%), and congenital scoliosis (7.5%) [Bijlsma et al., 2009; Fernandez et al., 2010; Rosenfeld et al., 2010; Walters et al., 2010; Wu et al., 2015]. *TBX6* encodes a transcription factor characterized by a T-Box DNA-binding domain. The gene plays a role in specification and patterning of somites as well as left/right patterning in mice [White et al., 2003; Hadjantonakis et al., 2008]. Besides vertebral and rib abnormalities, some homozygote hypomorphic mutations in Tbx6 are also associated with urogenital malformations [Nacke et al., 2000; White et al., 2003]. Depending on the genetic background, up to 60% of mice were affected with kidney malformations (renal aplasia/hypoplasia, malrotated, or displaced kidneys, hydronephrosis) or genital malformations (vaginal atresia or vagina duplex in females). Previously, *TBX6* was Sanger sequenced in a Finnish series of 112 females with MRKH [Sandbacka et al., 2013]. One heterozygous splice site variant was identified in two individuals. This variant is predicted to disrupt exon 5 splicing, which partially encodes for the T-box DNA-binding domain of the protein [Sandbacka et al., 2013]. Moreover, two rare single nucleotide polymorphisms (rs56098093 in exon 4 and rs201231713 in exon 6) were identified with a significantly higher frequency in females with MRKH (MAF = 8% and 5.8%, respectively) compared to controls (2% and 2%, respectively). Frequency of these variants were 0.5% (rs56098093) and 0.7% (rs201231713) in their Finnish cohort dataset and are 0.5% and 0.08% in the ExAC Browser [Exome Aggregation Consortium]. Two affected individuals were homozygous for both variants, nine were heterozygous for both variants and three were heterozygous for rs56098093. Although functional analyses were not performed, the two variants are located within the conserved T-Box domain and predicted to be damaging by four programs of in silico analysis (Polyphen 2, SIFT, Mutation Taster, and PROVEAN). In three individuals, a variant in *LHX1* or a copy number variant was also found. Overall, 20% of individuals in this series of 112 patients with MRKH presented a 16p11.2 deletion including *TBX6* or one or two rare variants in the gene, which might modify the transcription activity of the protein by haploinsufficiency or disruption of the T-Box DNA-binding domain. Recently, another study analyzed *TBX6* in a series of 167 individuals with MRKH or other Müllerian ducts abnormalities and identified the variant rs56098093 with a MAF of 1.5% (two carriers presented with MRKH type 2 and skeletal or renal anomalies and three presented with septate or sub-septate uterus). A 22q11.21 duplication was associated in one case as well as one rare missense variant in *TBX6* in an individual with a septate uterus. As part of this study, no variant was found in 70 individuals with MRKH type 1 [Tewes et al., 2015]. Pathways by which *TBX6* is involved in urogenital development still need to be clarified and the above data await reproduction in additional cohorts of individuals with uterine malformation. However, based on current data, *TBX6* might represent an important inherited susceptibility factor for congenital uterine malformations. In humans, *TBX6* has also been associated with segmental defects of vertebrae. A haploinsufficient stop-loss mutation was identified in a family with autosomal dominant spondylocostal dysostosis [Sparrow et al., 2013]. Compound heterozygosity of rare variants on a hypomorphic haplotype was suggested in another recent publication as a mechanism to explain 11% of congenital scoliosis [Wu et al., 2015]. Of note, vertebral anomalies are frequently reported in up to 49% of individuals with uterine malformations [McGowan et al., 2015]. Moreover, vertebral defects and uterine malformations have been associated in different overlapping conditions, including VACTERL association (vertebral defects, anal atresia, tracheoesophageal fistula with esophageal atresia, radial dysplasia, cardiac malformations, limb anomalies) [Orstavik et al., 1992; Linke et al., 2005; Komura et al., 2007; Nunes et al., 2009], caudal dysgenesis, OEIS (omphalocele–exstrophy–imperforate anus–spinal defects) complex, OAV (oculo-auriculo-vertebral) spectrum (aka Goldenhar syndrome or hemifacial microsomia) and MURCS association (Table IV). Causes for each of these conditions are still unknown and may be heterogeneous or multifactorial. Shared features of vertebral, Müllerian, and renal anomalies highlight the possibility of common genetic pathways involved in vertebrae segmentation and urogenital development, in which *TBX6* might serve as a key regulator.

The Wnt Signaling Pathways

Wnt pathways control several basic embryonic developmental processes including body axis patterning, cell fate specification, cell proliferation, and cell migration. It has long been recognized as one of the principal signaling pathways during development of mammalian urogenital tracts, mainly through the Wnt/β-catenin

TABLE IV. Syndromes Associated With Uterine Malformations

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
Multiple case reports—at least one case reported with a gene mutation					
Currarino syndrome (#176450)	Anorectal abnormalities, partial sacral agenesis, and presacral mass	Bicorporeal uterus, aplastic uterus, uterus duplication	O'Riordain et al. [1991], Lynch et al. [2000], Belloni et al. [2000], Martucciello et al. [2004], **Ciotti et al. [2011]**, Ohno et al. [2013]	***MNX1***	AD
Fraser syndrome (#219000)	Cryptophtalmos, nose and ear anomalies, laryngeal stenosis, renal agenesis/ hypoplasia, intellectual disability, ambiguous external genitalia	Bicorporeal uterus, hemi-uterus, aplastic uterus	Slavotinek and Tifft [2002], **Slavotinek et al. [2006]**, **Shafeghati et al. [2008]**, Kalaniti and Sandhya [2011]	***FRAS1, FREM***, GRIP1	AR
Hand–foot–genital syndrome (#140000)	Small feet with short great toes and abnormal thumbs, genital anomalies, ectopic accessory ureteral orifice, vesicoureteral reflux, ureteropelvic junction obstruction	Various degrees of bicorporeal uterus with or without two cervices or a longitudinal vaginal septum	**Goodman et al. [2000]**, **Mortlock and Innis [1997]**, **Innis et al. [2004]**, **Utsch et al. [2007]**, **Jorgensen et al. [2010]**, **Parker et al. [2011]**, **Imagawa et al. [2014]**	***HOXA13***	AD
HDR (hypoparathyroidism, sensorineural deafness and renal anomaly) syndrome (#146255)	Symptomatic or asymptomatic hypocalcemia, undetectable or low level of serum parathyroid hormone (PTH), bilateral sensorineural deafness, kidney anomalies (cysts, hypoplasia/aplasia), vesicoureteral reflux, glomerular nephropathy, and nephrotic syndrome	Bicorporeal uterus, aplastic uterus, septate uterus	**Nakamura et al. [2011]**, **Moldovan et al. [2011]**, **Hernández et al. [2007]**	***GATA3***	AD
Müllerian aplasia and hyperandrogenism (#158330)	Hyperandrogenism, pelvic kidney, renal agenesis	Aplastic uterus	**Philibert et al. [2011, 2008]**, **Biason-Lauber et al. [2007, 2004]**	***WNT4***	AD
Renal cysts and diabetes syndrome (#137920)	Abnormal liver function, early-onset diabete mellitus, hypomagnesemia, pancreatic hypoplasia, hyperuricemia, congenital anomalies of the kidney, and urinary tract	Aplastic uterus, bicorporeal uterus	**Bingham et al. [2002]**, **Shihara et al. [2004]**, **Harries et al. [2005]**, **Edghill et al. [2006, 2008]**, **Oram et al. [2010]**, **Iwasaki et al. [2001]**, **Lindner et al. [1999]**,	***HNF1B***	AD

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
			Carbone et al. [2002]		
Smith–Lemli–Opitz (#270400)	Prenatal and postnatal growth retardation, microcephaly, intellectual disability, distinctive facial features, cleft palate, cardiac defects, underdeveloped external genitalia in males, postaxial polydactyly, 2–3 syndactyly of the toes	Bicorporeal uterus	**Quélin et al. [2012]**, Lowry et al. [1968]	***DHCR7***	AR
STAR syndrome (toe syndactyly, telecanthus, anogenital and renal malformations syndrome) (#300707)	Telecanthus, clinodactyly 5th finger, syndactyly of toes, anal atresia, genital anomalies (hypolastic labia, duplicated vagina), VU reflux, kidney agenesis, small height.	Bicorporeal uterus, duplicated uterus	**Unger et al. [2008]**	***FAM58A***	X-linked
Syndromic ciliopathies (#616258, #236700, #236680, #209900)	Bardet–Biedel: intellectual disability, pigmentary retinopathy, polydactyly, obesity, hypogonadotropic hypogonadism	Aplastic uterus, bicorporeal uterus, uterine hypoplasia	**Filges et al. [2014]** (fetal ciliopathy-MKS12)	***KIF14, TCTN3, BBS12***[a]	AR
	McKusick–Kaufman: polydactyly, genitourinary malformation (hydrometrocolpos-transverse vaginal septum), heart and gastro-intestinal malformations		Witters et al. [2012] (MKKS)		
	Hydrocephalus: hydrocephaly, micrognathia, cleft lip/palate, malformed respiratory tract, congenital heart defects, club feet, polydactyly		**Thomas et al. [2012]** (fetal ciliopathy with feature of MKS and OFDIV)		
	Meckel syndrome: encephalocele, postaxial polydactyly, dysplastic polycystic kidneys, male pseudohermaphrodism		**Deveault et al. [2011]** (BBS-(vaginal agenesis))		
	Meckel syndrome 12: intrauterine growth retardation, severe microcephaly, renal cystic		Salonen and Herva [1990] (hydrolethalus)		

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
	dysplasia/agenesis, and complex brain and genitourinary malformations				
	Mohr–Majewski syndrome (OFD IV): bone dysplasia, tibial defect, renal anomalies, brain anomalies		Stoler et al. [1995], Campo and Aaberg [1982] (BBS)		
Syndromic microphtalmia types 12 and 9 (Matthew-wood syndrome; PDAC syndrome) (#615524; #601186)	Severe pulmonary aplasia or dysgenesis or hypoplasia, diaphragmatic eventration or hernia, anophthalmia or microphthalmia or ocular dysplasia, cardiac defect.	Bicorporeal uterus, hypoplastic uterus	Chitayat et al. [2007], **Srour et al. [2013]**	*STRA6*, ***RARB***	AR, AD
SPECC1L hypertelorism syndrome (Opitz G/BBB syndrome type II; Teebi hypertelorism syndrome) (#145410; %145420)	Hypertelorism, umbilical hernia, craniosynostosis, cleft lip and palate, prominent forehead, broad nasal root, aortic root dilation	Bicorporeal uterus	**Bhoj et al. [2015], Kruszka et al. [2015]**	***SPECC1L***	AD
Townes–**Brocks (#107480)**	Imperforate anus, dysplastic ears (overfolded superior helices and preauricular tags; frequently associated with sensorineural and/or conductive hearing impairment), thumb malformations, renal anomalies	Bicorporeal uterus, septate uterus	**Botzenhart et al. [2005]**, Johnson et al. [1996]	***SALL1***	AD
Ulnar and fibula syndrome, with severe limb deficiency (#276820)	Absence or reduction of limbs, facial abnormalities, pelvic and genital abnormalities, horseshoe kidneys, unilateral renal agenesis, hydronephrosis	Aplastic uterus	**Eyaid et al. [2011]**, **Al-Qattan et al. [2013]**, Farag et al. [1993], Teebi [1993]	***WNT7A***	AR
Woodhouse–Sakati syndrome (#241080)	Alopecia, diabetes mellitus, hypogonadism, deafness, cognitive decline, extrapyramidal features	Aplastic uterus, uterine hypoplasia	**Steindl et al. [2010]**	***C2ORF37***	AR
		Single case report with an identified gene			
Cenani–Lenz syndrome (prenatal lethal form) (#212780)	Syndactyly and oligodactyly, partial to complete fusion of the carpals, metacarpals, or phalanges,	Aplastic uterus	**Lindy et al. [2014]**	***LRP4***, APC	AR

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
	radioulnar synostosis, mesomelic shortening, prominent forehead, hypertelorism, downslanting palpebral fissures, and micrognathia, renal agenesis/hypoplasia				
Chondrodysplasia, acromelic, with genital anomalies (#609441)	Short stature, acromesomelic shortness of the limbs, hand/foot malformations, absence of ovaries	Aplastic uterus	**Demirhan et al. [2005]**	***BMPR1B***	AR
Cousin syndrome (#260660)	Short stature, short limb, macrocephaly (real or relative), dysmorphic features, humeroradial synostosis, hips dislocation, scapular and iliac hypoplasia	Arcuate uterus	**Dikoglu et al. [2013]**	***TBX15***	AR
Lymphoedema–distichiasis syndrome (#153400)	Distichiasis, primary lymphoedema of pubertal onset	Double uterus	**Brice et al. [2002]**	***FOXC2***	AD
Limb mammary syndrome (#603543)	Ectrodactyly, mammary-gland and nipple hypoplasia, cleft palate without cleft lip	Aplastic uterus	**Guazzarotti et al. [2008]**	***TP63***	AD
Leukodystrophy, hypomyelinating, 2 (#608804)	Nystagmus, impaired motor development, ataxia, choreoathetotic movements, dysarthria, and progressive spasticity, renal agenesis	Aplastic uterus	**Yalcinkaya et al. [2012]**	***GJC2***	AR
Serpentine fibula polycystic kidneys (Hajdu–Cheney syndrome) (#102500)	Craniofacial abnormalities (prominent eyes, full cheeks, arched eyebrows, and micrognathia), skeletal abnormalities (wormian bones, fibular bowing, and osteoporosis), polycystic kidneys	Bicorporeal uterus	**Martin et al. [2014, 2]**	***NOTCH2***	AD
SOPH (short stature, optic nerve atrophy, and Pelger–Huet anomaly) syndrome (#614800)	Postnatal growth failure, loss of visual acuity, Pelger–Huët anomaly, craniofacial features (Yakuts population)	Hypoplastic uterus	**Maksimova et al. [2010]**	***NBAS***	AR
Tetra-amelia syndrome, autosomal recessive (#273395)	Tetra-amelia, cleft lip/palate, gastroschisis,	Malformed uterus	**Niemann et al. [2004]**	***WNT3***	AR

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
	diaphragmatic defect, lung anomaly, adrenal gland agenesis, spleen agenesis, ophtalmological anomalies, malformed nose, choanal atresia, atresia of urethra, vagina and anus, hypoplasia of pelvis, abnormal externe genitalia, renal agenesis				
Ulnar-mammary syndrome (#181450)	Posterior limb deficiencies or duplications, apocrine/ mammary gland hypoplasia and/or dysfunction, abnormal dentition, delayed puberty in males, genital anomalies	Bicorporeal uterus	**Sasaki et al. [2002]**	***TBX3***	AD
	≥2 case reports not investigated molecularly (but etiology known for the syndrome)				
Apert syndrome (#101200)	Craniosynostosis, midface hypoplasia, finger and toe anomalies and/or syndactyly	Bicorporeal uterus	Blank [1960], Lindsay et al. [1975]	*FGFR2*	AD
Basal cell nevus syndrome (Gorlin syndrome) (#109400)	Multiple early-onset basal cell carcinoma (BCC), odontogenic keratocysts, skeletal abnormalities	Bicorporeal uterus, septate uterus, infantilis uterus/ uterine hypoplasia	Kimonis et al. [1997], Ramaglia et al. [2006], Kohli et al. [2010]	*PTCH1, PTCH2, SUFU*	AD
Beckwith–Wiedemann syndrome (#130650)	Macrosomia, macroglossia, visceromegaly, embryonal tumors, omphalocele, neonatal hypoglycemia, ear creases/pits, adrenocortical cytomegaly, and renal abnormalities.	Bicorporeal uterus	Weng et al. [1995], Irving [1967]	*Loss of methylation IC2 (maternal allele); paternal UPD of 11p15; gain of methylation IC1 (maternal allele); CDKN1C mutations*	AD
Coffin–Siris syndrome (#135900)	Aplasia or hypoplasia of the distal phalanx or nail of the fifth digit, distinctive facial features, moderate to severe developmental/ cognitive delay	Aplastic uterus	Goyal et al. [2010] Coffin and Siris [1985]	*ARID1A, ARID1B, SMARCA4, SMARCB1, or SMARCE1*	*AD*
Goltz syndrome (#305600)	Focal dermal hypoplasia, asymetric limbs anomalies, dental, nails and hair anomalies, asymetry of face, dysmorphic features,	Bicorporeal uterus	Lopez-Porras et al. [2011], Reddy and Laufer [2009]	*PORCN*	X-linked

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
	eyes anomalies, kidney anomalies				
Linear skin defects with multiple congenital anomalies 1 (MIDAS syndrome) (#309801)	Unilateral or bilateral microphthalmia, linear skin defects (aplastic skin, hyperpigmented area) limited to the face and neck	Bicorporeal uterus, rudimentary uterus	Happle et al. [1993], Anguiano et al. [2003]	*HCCS*	X-linked
Neu–Laxova syndrome (#256520)	Severe fetal growth restriction, microcephaly, distinct facial appearance, ichthyosis, skeletal anomalies, perinatal lethality	Bicorporeal uterus, hemi-uterus	Coto-Puckett et al. [2010], Ostrovskaya and Lazjuk [1988]	*PHGDH*	AR
Peters–Plus syndrome (#261540)	Anterior chamber eye anomalies, short limbs with broad distal extremities, variable developmental delay/ intellectual disability, exaggerated Cupid's bow of the upper lip, short palpebral fissures, ear anomalies, cleft lip/palate.	Rudimentary uterus	Thompson et al. [1993], Streeten et al. [1983], Maillette de Buy Wenniger-Prick and Hennekam [2002]	*B3GALTL*	AR
Popliteal pterygium syndrome (#119500)	Cleft lip, a cleft palate, fistulae of the lower lip, Webbing of the skin extending from the ischial tuberosities to the heels, bifid scrotum and cryptorchidism, hypoplasia of the labia majora, syndactyly of fingers and/ or toes, anomalies of the skin around the nails	Bicorporeal uterus, hypoplastic uterus	Bartsocas and Papas [1972], Gorlin et al. [1968]	*IRF6*	AD
TAR syndrome (#274000)	Bilateral absence of the radii with presence of both thumbs, transient thrombocytopenia, kidney anomalies	Aplastic uterus	Griesinger et al. [2005], Behera et al. [2005], Childs and Hall [2005], Ahmad and Pope [2008], Rall et al. [2015b]	Del1q21+ *RBM8A*	AR
Chromosomal syndromes (≥2 case reports)					
22q11.2 deletion (#188400)	Growth retardation, intellectual disability or learning difficulties	Aplastic uterus	Cheroki et al. [2006], Devriendt et al. [1997], Sundaram et al. [2007],	Chromosomal	AD

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
			Uliana et al. [2008], Morcel et al. [2011]		
17q12 deletion (#614527)	Developmental delay, learning disability, autism or autistic features, kidney and urinary tract problems, diabetes, behaviour concerns, facial features	Aplastic uterus, bicorporeal uterus	Cheroki et al. [2008], Nik-Zainal et al. [2011], Bernardini et al. [2009], Moreno-De-Luca et al. [2010]	Chromosomal	AD
Cat-eye syndrome (#115470)	Anal atresia, iris coloboma, downward-slanting palpebral fissures, preauricular pits/tags, cardiac defects, kidney problems, short stature, scoliosis/skeletal problems, intellectual disability, micrognathia, cleft palate	Aplastic uterus	Cullen et al. [1993], Schinzel et al. [1981]	Chromosomal (trisomy or tetrasomy 22pter-22q11)	AD
Pallister–Killian syndrome (#601803)	Profound intellectual disability, seizures, streaks of hypo- or hyper-pigmentation, facial anomalies (prominent forehead with sparse anterior scalp hair, flat occiput, hypertelorism, short nose with anteverted nostrils, flat nasal bridge, short neck)	Aplastic uterus, hypoplastic bicorporeal uterus	Reynolds et al. [1987], Bernert et al. [1992]	Chromosomal (Tetrasomy 12p mosaic)	Somatic mosaicism
Turner syndrome	Short stature, gonadal dysgenesis, cardiovascular malformations, oedema of the hands and feet, renal anomalies	Rudimentary uterus, infantilis uterus/uterine hypoplasia, aplastic uterus	Wonkam et al. [2015], Vaddadi et al. [2013], Bakalov et al. [2007]	Chromosomal (45,X)	Sporadic
Trisomy 13 (Patau syndrome) Microcephaly, microphthalmia, malformed ears, cleft lip and palate, cardiac anomalies, polydactyly, intrauterine growth retardation, severe developmental retardation	Holoprosencephaly, microphtalmia/ anophtalmia, cutis aplasia, cleft lip/palate, cardiac malformation, postaxial polydactyly, omphalocele, kidney malformations, severe/profound intellectual disability, growth retardation.	Bicorporeal uterus, hemi-uterus, septate uterus, arcuate uterus	Moerman et al. [1988], Baty et al. [1994]	Chromosomal	Sporadic
Trisomy 18 (Edwards' syndrome)	Intrauterine growth retardation, congenital	Bicorporeal uterus	Baty et al. [1994], Kinoshita et al. [1989]	Chromosomal	Sporadic

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
	heart defect, renal defects, short sternum, prominent occiput, micrognathia, malformed ears, overlapping fingers, severe to profound developmental delay				
Wolf–Hirschhorn syndrome (#194190)	Pre- and postnatal growth deficiency, developmental disability, characteristic craniofacial features ('Greek warrior helmet' appearance of the nose), seizure disorder	Aplastic, hypoplastic uterus	Johnson et al. [1976]	Chromosomal (deletion 4p16.3)	Sporadic
Syndromes and associations of unknown etiology (≥3 cases reported)					
Acro-renal-mandibular syndrome (200980)	Ectrodactyly, limb deficiency, mandibular hypoplasia, bilateral renal agenesis, cystic dysplasia	Bicorporeal uterus, hemi-uterus	Sanganalmatha et al. [2014], Evans et al. [2000], Halal et al. [1980]	/	/
Caudal duplication syndrome (#607864)	Varied extent of duplication of the colon, rectum, anus, urinary system, lower genital tract, and external genitalia; spinal anomalies; kidney anomalies; abdominal wall defects	Duplication of the female genital tract	Acer et al. [2013], Shah and Joshi [2006], Dominguez et al. [1993]	*AXIN1 (hypermethylation of the promotor in 1 case)*	/
Caudal dysgenesis spectrum (including sirenomelia) (#600145)	Variable association of anomalies of the caudal spine and spinal cord, the hindgut, the urogenital system, and the lower limbs.	Bicorporeal uterus, aplastic uterus	Tica et al. [2013] (Sirenomelia[b]), Harewood et al. [2010] (caudal dysgenesis), Bashyal et al. [2012] (pelvis dysplasia-urogenital abnormalities)	*VANGL1*	/
Fryns syndrome (229850)	Coarse face, abnormal ear shape, CL/CP, large mouth, microretrognathia, broad nasal bridge, anteverted nares, diaphragmatic defects, distal digital hypoplasia, genital tract anomalies, malformation CNS, abnormal lung lobations, cystic dysplasia of kidneys	Bicorporeal uterus	Slavotinek [2004]	/	/

TABLE IV. (*Continued*)

Syndrome (OMIM identification)	Features	Uterine anomalies	Clinical case reports (literature)	Genes	Inheritance
Hypergonadotrophic hypogonadism and partial alopecia (241090)	Hypergonadotrophic hypogonadism, partial alopecia, abnormal gonads (streak ovaries, absence)	Aplastic/hypoplastic uterus	Al-Awadi et al. [1985], Mégarbané et al. [2003], Tatar et al. [2009]	/	/
MURCS association (601076)	cervicothoracic somite dysplasia, renal aplasia, ectopic kidney	Aplastic uterus, bicorporeal uterus	Braun-Quentin et al. [1996]	/	/
Oculo-auriculo-vertebral complex (Hemifacial microsomia, Goldenhar syndrome) (164210)	Hypoplastic malar, maxillary and mandibular regions, microtia, hemivertebrae, hypoplastic vertebrae, kidney and urinary tract anomalies	Aplastic uterus	Wulfsberg and Grigbsy [1990], Pillay et al. [2003]	/	/
OEIS complex (258040)	Omphalocele, exstrophy of the cloaca, imperforate anus, and spinal defects, ambiguous genitalis, kidney anomalies	Bicorporeal uterus	Dutta [2014], Sawaya et al. [2010]	/	/
Pseudotrisomy 13 syndrome (264480)	Holoprosencephaly, severe facial anomalies, postaxial polydactyly, heart defect	Bicorporeal uterus, duplicated uterus, hypoplastic uterus	Ramos-Arroyo et al. [1994]	/	
Schinzel-phocomelia (276820)	Skull defect, phocomelia, vesicoureteral reflux	Aplastic uterus	Schinzel [1990], Froster et al. [1996], Chitayat et al. [1993]	/	/
Spondylocostal dysostosis (Casamassima–Morton–Nance syndrome) (271520)	Spondylocostal dysostosis, anal atresia and urogenital anomalies (absence of vagina and external genitalia)	Bicorporeal uterus, aplastic uterus	Poor et al. [1983], Daïkha-Dahmane et al. [1998], Thauvin-Robinet et al. [2007], Sasaki et al. [2011]	/	/
VATER/VACTERL association (#192350)	Vertebral defects, anal atresia, tracheoesophageal fistula with esophageal atresia, radial dysplasia, cardiac malformations, limb anomalies, kidney anomalies	Aplastic uterus, hemi-uterus	Linke et al. [2005], Orstavik et al. [1992], Komura et al. [2007], Nunes et al. [2009], Jessel and Laufer [2013]	*HOXD13* (one case)	/

Review of the literature based on OMIM database, the Winter–Baraitser Dysmorphology Database, and Smith's Recognizable Patterns of Human Malformation: Expert Consult—Online and Print, 7e, 2013.
References in bold when some mutations were reported; genes in bold when mutations have been reported in some individuals with uterine malformations.
[a]Sixty-nine genes have been indexed in OMIM as associated with human ciliopathies.
[b]Anti-epileptic drugs during the pregnancy.

signaling pathway with particular contributions from Wnt9b and Wnt4 [Carroll et al., 2005; Halt and Vainio, 2014]. During the first step of Müllerian duct development, invagination of coelomic epithelial cells requires Wnt4 signaling, and absence of expression leads to Müllerian duct agenesis in both male and female mice embryos [Vainio et al., 1999]. Wnt4 is also expressed by mesenchymal cells surrounding the Müllerian ducts [Vainio et al., 1999], suggesting a role in further developmental steps [van der Horst et al., 2012]. Wnt9b, expressed by Wolffian duct cells, is involved subsequently, and paracrine signaling from these cells is necessary for Müllerian duct elongation. Wnt9b knockout female mice lack the oviduct, uterus, and upper part of the vagina [Carroll et al., 2005]. Gonads are normal in $Wnt9b^{-/-}$ mice, whereas ovaries in $Wnt4^{-/-}$ female mice are abnormal with a testis-like appearance. Both genes are furthermore involved in induction of metanephric kidneys such that knockout mice develop rudimentary kidneys. Wnt9b acts upstream of Wnt4 and is likewise indispensable for mesonephric tubule development [Carroll et al., 2005]. Later nephrogenesis, medullary differentiation, and collecting duct development involve Wnt9b, Wnt4 as well as additional Wnt ligands and receptors from the Wnt pathway (i.e., Wnt11, Wnt7b, and Wnt5a). Wnt5a mutations in mice have recently been implicated in duplex collecting system malformations via the non-canonical Wnt/PCP signaling pathway [Yun et al., 2014]. In uterine development and differentiation, two other members of the Wnt pathway have been shown to play an important role. Wnt7a, secreted by Müllerian duct epithelial cells, is involved in the patterning of Müllerian ducts. Posteriorization of the FGT is noted in $Wnt7a^{-/-}$ mice, usually with absence of oviducts and a vaginal-like histological appearance of the uterus [van der Horst et al., 2012]. Wnt5a, secreted by mesenchymal cells, is necessary for posterior development of the FGT as knockout mice lack cervical and vaginal structures. Both Wnt7a and Wnt5a are furthermore involved in uterine gland development within the endometrium [Massé et al., 2009]. Wnt genes participate in the development of other organs as well. For example, Wnt7a plays a role in limb development and Wnt9b knockout mice present variably penetrant cleft lip and palate.

In humans, *WNT4* was the first gene from the Wnt pathway to be associated with uterine malformations. Affected women with missense mutations in this gene have also presented with biochemical and clinical features of hyperandrogenism. Three individuals with likely pathogenic variants and one with a variant of unknown significance have been reported so far, and absence of variants in some individuals with MRKH and hyperandrogenism also suggests the involvement of other proteins in the WNT4/β-catenin signaling pathway [Philibert et al., 2011]. A dominant negative effect has been suggested by in vitro functional analysis of these variants [Biason-Lauber et al., 2004, 2007; Philibert et al., 2008]. Only two individuals with isolated uterine malformations (among 189 and 11 women with Müllerian defects and MRKH, respectively) had rare variants in *WNT4*. These variants are synonymous and were not proven to affect splicing or function [Ravel et al., 2009; Chang et al., 2012]. Another variant was found in an individual with renal hypodysplasia [Vivante et al., 2013], and a homozygous mutation has been reported in a family with female sex reversal, dysgenesis of kidneys, adrenals, and lungs (SERKAL syndrome) [Mandel et al., 2008].

WNT7A homozygous mutations were first associated in 2006 with a range of limb malformations, including the phenotypes of Al-Awadi/Raas-Rothschild (AARR) syndrome (characterized by apparent phocomelia, hypoplastic pelvis, and abnormal genitalia) and Fuhrmann syndrome (considered an attenuated phenotype of AARR) [Woods et al., 2006]. Two patients with AARR syndrome and different *WNT7A* homozygous missense mutations in exon 4 were reported with uterine hypoplasia [Eyaid et al., 2011; Al-Qattan et al., 2013]. The uterine phenotype was unknown or not reported for other affected female individuals [Woods et al., 2006; Kantaputra et al., 2010; Eyaid et al., 2011] and several individuals with uterine anomalies were reported with AARR syndrome or Schinzel phocomelia (a syndrome controversially grouped with AARR syndrome in OMIM) before the gene discovery [Schinzel, 1990; Chitayat et al., 1993; Farag et al., 1993; Teebi, 1993; Froster et al., 1996]. Beyond this easily recognizable condition, *WNT7A* has been tested in three series of patients with MRKH or Müllerian anomalies [Timmreck et al., 2003b; Ravel et al., 2009; Dang et al., 2012] and rare heterozygous synonymous variants have been found in two cases of isolated Müllerian anomalies. Although these variants are synonymous, they were predicted to be disease-causing by Mutation Taster and to potentially alter splicing by Human Splicing Finder. However, alteration of splicing was not proven with mRNA analysis.

WNT9B has been analyzed in two series (n = 11 and 42) of individuals with MRKH syndrome [Ravel et al., 2009; Wang et al., 2014] and in two series of 191 and 226 individuals with a broader spectrum of Müllerian anomalies [Tang et al., 2014; Waschk et al., 2015]. Eight variants of unknown significance were identified in affected individuals and absent in study controls. In the large Caucasian cohort, five heterozygous missense variants were identified as well as one heterozygous nonsense variant located at the 3′ end of the gene. No rare, damaging variants were identified in the control cohort, implicating this gene with statistical significance. The phenotypes were a bicorporeal uterus for one individual and isolated MRKH syndromes for the five others. According to ACMG variant interpretation guidelines, further evidence, such as functional data, would be necessary to consider any of these individual variants as likely pathogenic. Based on these results, *WNT9B* might be involved in up to 8.5% of women with isolated MRKH, possibly in a polygenic manner with co-inheritance of other genetic factors such as *TBX6* mutation or *LHX1* deletion [Waschk et al., 2015].

Finally, one individual with a bicorporeal uterus among 189 females with uterine malformations was identified with an intronic variant of uncertain significance in *WNT5A* which was absent in 198 controls [Wu et al., 2013].

To date, the pathologic significance of the individual variants identified is unknown, but it is clear that alteration of the Wnt pathway can predispose to uterine malformation.

Other Candidate Genes From Copy Number Variants

Other candidate genes have been suggested from analyses using microarray or multiplex ligation-dependent probe amplification in

individuals with MRKH. *PAX8* is a transcription factor of the paired-box family that has been associated with isolated and syndromic thyroid dysgenesis [Park and Chatterjee, 2005]. This gene is located within a 10.79 Mb deletion of 2q13q14.2 identified in a girl presenting with hypothyroidism, developmental delay, scoliosis, dysmorphic features, and a hemi-uterus. Pax8 homozygote knockout mice, supplemented with synthetic T4, are infertile due to uterine aplasia/hypoplasia and lack of a vaginal opening [Mittag et al., 2007]. During murine development, Pax8 expression is detected in epithelium of the uterus, oviduct, and vagina [Mittag et al., 2007]. Pax8, with Pax2, is also a central regulator of kidney development and acts upstream of Lim1 and Gata3 [Marcotte et al., 2014]. Renal agenesis, without uterine malformation, has been reported in individuals with syndromic *PAX8* mutations [Park and Chatterjee, 2005].

In a series of 30 individuals with MRKH syndrome, intragenic partial duplications of *SHOX* (short stature homeobox), spanning 7–300 kb, were identified in four unrelated individuals with isolated MRKH. In one familial case, the duplication was also found in an affected sister and healthy father [Gervasini et al., 2010]. However, these results were somewhat controverted by absence of any intragenic duplication in *SHOX* identified in a larger cohort of 101 individuals with MRKH [Sandbacka et al., 2011]. To our knowledge, this gene has not yet been systematically assessed for point mutations in a similar cohort. Furthermore, intragenic duplications have been reported in individuals with idiopathic short stature and Léri-Weill dyschondrosteosis without mention of any uterine anomalies [Benito-Sanz et al., 2011]. *SHOX*, located in Xp22.33 in the pseudoautosomal region of chromosome X, belongs to the paired homeobox family. The gene does not have any equivalent in rodents and whereas *SHOXb* transcript is expressed in the fetal kidneys [Rao et al., 1997], expression in fetal Müllerian ducts has not been proven. Whether *SHOX* plays a role in uterine development and whether intragenic duplications of *SHOX* truly confer a risk for uterine malformations remains to be determined.

ITIH5 has been suggested as another potential contributor based on microarray results [Morcel et al., 2012a]. A de novo deletion in 10p14 causing sole loss of *ITIH5* was identified in a fetus with MRKH anomaly and bilateral renal agenesis. However, the deletion was not found in the more mildly affected relatives with uterine or renal anomalies [Morcel et al., 2011]. *ITIH5*, a member of the inter-alpha-trypsin inhibitor (ITI) family, was shown to be expressed in the cranial portion of the Müllerian ducts during mouse embryogenesis [Morcel et al., 2012b] and was hypothesized to contribute, as a modifier to undetected factors, to the more severe phenotype in the proband.

Finally, deletions and duplications in 22q11.2, both proximal and distal, are associated with a wide range of anomalies. MRKH anomaly, isolated or in association with other symptoms, can be part of the phenotype [Devriendt et al., 1997; Cheroki et al., 2006; Sundaram et al., 2007; Cheroki et al., 2008; Uliana et al., 2008; Ledig et al., 2011; Morcel et al., 2011; Nik-Zainal et al., 2011]. However, causal genes within these regions that are involved in genital development remain to be determined. Single associations usually of uncertain clinical significance have been reported for several other chromosomal regions.

Syndromes With Uterine Malformations and Known Genetic Cause

Following the observation of recurrent uterine malformations in RCAD syndrome, HFG syndrome, and AARR syndrome, sequencing of the causative genes, *HNF1B*, *HOXA13*, *WNT7A*, respectively, was undertaken in individuals with isolated uterine malformations without, to date, any significant results (as reviewed above). Uterine agenesis and uterine malformations have been reported in other Mendelian syndromes or associations (Table IV), and these other genes might be worth examining in cohorts with isolated uterine anomalies. For example, uterine malformations are frequently reported in Fraser syndrome. Mild bi-allelic mutations in Fraser syndrome genes (*FRAS1*, *FREM2*, *GRIP1*, and *FREM1*) were identified in individuals with isolated CAKUT [Kohl et al., 2014] and underlined the possibility of mild and incomplete Fraser syndrome phenotypes associated with these genes. *LRP4*, *PORCN*, and *WNT3* are members of the Wnt signaling family. Considering ciliopathies as one pathological group with different overlapping phenotypes, uterine malformations emerge as a recurrent phenotypic feature, which is not surprising given the role of the cilia in transducing developmental signals—in particular, the Wnt pathway [May-Simera and Kelley, 2012].

NEGATIVE RESULTS

Large series of individuals have been sequenced for many genes with a relatively low rate of compelling genetic variation (Table III). Recently, the first research study combining next-generation sequencing and SNP array in a small series (five individuals with isolated MRKH) did not identify any convincing common causative genes [Chen et al., 2015]. Functional evidence is difficult to build for particular variants in developmental genes considering the confined temporospatial expression of many of these genes. Since the aggregation of very large population cohorts (e.g., as compiled in ExAc), it has become easier to rule out variants as having large independent effects when they are recurrent in the background population. The difficulty to date in identifying genetic causes for uterine malformations may have several explanations. First, our current understanding of genes and pathways involved in uterine development is partial, and approaches to date have mostly focused on candidate genes analyzed by targeted methodologies such as Sanger sequencing. Of note, comparisons to animal models are made difficult by differences in uterine development in humans and other species [Kobayashi and Behringer, 2003], especially when studying fusion and absorption septal defects. Next-generation sequencing in larger cohorts may be the best approach at present to identify novel causal genes.

Second, uterine malformations may be highly genetically heterogeneous in their etiology with single genes each explaining only a few occurrences. Alternatively, these anomalies may be shown to be polygenic in origin, such that a combination of variants in the same regulating pathway might be required for penetrance of the phenotype. Association studies, which may help to identify susceptibility variants, have been rarely carried out in cohorts with uterine malformations. Regarding MRKH syndrome in a Han Chinese population, Ma et al. [2015] recently reported that the

variants rs34072914 (c.399G>T) in *WNT9B* and c.934C>T in *AMH* were associated with non-isolated MRKH and isolated MRKH, respectively [Ma et al., 2015]. They reported epistatic effects for these variants when accompanied by other SNPs in a set of candidate genes. Replication will be necessary to prove this potential association, with consideration given to the potential contribution of ethnic background.

Third, the possibility of somatic mosaicism for de novo mutations is suggested by discordance in monozygotic twins and absence of recurrence in offspring of affected women (which could be explained theoretically by lethality in a germline). Among five pairs of MZ twins, differences in copy number variants across tissues were demonstrated in one affected twin of a pair and included duplication of *LRP10*, an inhibitor of the canonical Wnt pathway, and *MMP14*, a matrix metalloproteinase that might play a role in Müllerian ducts cell migration [Rall et al., 2015a].

Finally, besides genetic factors, evidence exists for the involvement of environmental factors. In humans, the best-known example is the association of uterine anatomical deviations (i.e., small endometrial cavities, T-shaped uteri or dilated cornuate areas) with prenatal exposure to DES (diethylstilbestrol), a synthetic estrogen widely used to treat threatened abortion from 1940 to 1980. In mice, exposure to DES has been shown to shift the pattern of expression of Hoxa9–11 to a more caudal distribution and to induce an anterior transformation of the reproductive tract [Block et al., 2000]. The T-shaped uteri in humans may reflect HOXA9 overexpression in the uterus, leading to an oviduct like phenotype [Block et al., 2000]. DES is considered an endocrine-disrupting chemical (EDC) and, like other chemicals such as bisphenol A, phthalates or pesticides, it has been proven to interfere with endocrine systems in mammals [Costa et al., 2014]. Binding to estrogen nuclear receptors and altering regulatory regions of developmental genes by epigenetic modifications, EDCs may regulate transcription of genes essential for development of the uterus. Exposure during a critical period of development may disturb normal genital tract formation [Yamashita, 2006]. For instance, in mice, Hoxa10 transcription was altered following DES or BPA exposure [Block et al., 2000; Smith and Taylor, 2007], and epigenetic modifications of the Hoxa10 promoter were noted after BPA exposure [Bromer et al., 2010]. Expressions of other genes (eg., Wnt7a, Vegf, and Msx2) have been shown to be altered after DES exposure [Huang et al., 2005; Yamashita, 2006; Suzuki et al., 2007]. It is currently unknown whether other EDCs may also cause uterine malformations in the range of doses to which human fetuses might be exposed. In rats, exposure to high dose of phthalates (520 mg/kg/day mixture of five different phthalates between the 8th to the 19th day of gestation) has been recently associated with uterine malformations (i.e., uterus agenesis, hemi-uterus, absence of vaginal opening) and suggested as a promising animal model to study pathogenesis of MRKH syndrome [Hannas et al., 2013].

Epigenetic alterations of developmental genes, secondary to environmental factors or genetic causes, can be potentially limited to one organ or tissue. Epigenetic causes could also explain sporadic cases of uterine malformations and MRKH syndrome as well as discrepancy observed between MZ twins. Comparison of transcription and methylation profiles in post-surgery uterus myometrium from adult individuals with MRKH and controls highlighted differences in both transcription and methylation for six genes potentially relevant to the development of female reproductive tracts (*CDH5*, *MFAP5*, *WISP2*, *HOXA5*, *PEG10*, *HOXA9*) [Rall et al., 2011]. However, further studies are necessary to determine links between epigenetic modifications of developmental genes and uterine development as well as how environmental factors are involved in this process.

CONCLUSION AND CURRENT PERSPECTIVE

The ability to molecularly diagnose individuals with MRKH syndrome or uterine malformations is currently very limited. Recurrence risk for siblings or offspring is best estimated at 1–3%, in the absence of a positive family history. Chromosomal microarray could be offered to those with MRKH syndrome, those with uterine malformations and an additional malformation, or those with a positive family history, although confident prediction of penetrance for most of the relevant copy number variants is not currently possible. The family history should include inquiry regarding renal or vertebral malformations and diabetes. *HNF1B* testing should be offered to individuals with uterine malformations and either/both MODY or renal anomalies, or a family history of such, particularly because the risk of renal disease and MODY may influence clinical management and because a positive result would modify the genetic counseling. Deployment of next-generation sequencing technologies undoubtedly will identify novel genes and provide new understanding of uterine development and diagnostic testing options for individuals. Compelling hypotheses to be explored include pathway-based polygenicity, somatic mosaicism, and epigenetic alterations.

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