Identification of novel genes and mechanisms responsible for recurrent pregnancy loss

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ABSTRACT

Approximately 10-20% of clinically recognized pregnancies result in spontaneous loss. The occurrence of at least two of such events before 24 weeks of gestation is termed recurrent pregnancy loss (RPL), which affects 1-5% of couples trying to conceive, and approximately half of these cases remain clinically unexplained. Exome sequencing on patients with RPL revealed a homozygous nonsense mutation in *HORMAD2* in a patient with eight recurrent miscarriages and no live birth. HORMAD2 is an essential protein of the synaptonemal complex. Microscopic morphological evaluation of one of the patient's miscarriages confirmed its diagnosis. Microsatellite genotyping on available DNA from two other miscarriages demonstrated that they are triploid digynic and resulted from the failure of maternal Meiosis II (MII). Single nucleotide polymorphism (SNP) microarray analysis revealed an additional Meiosis I (MI) abnormality that is the segregation of the two maternal homologous chromosomes 16 and 19 in one conception. My data will improve current understanding of the genetic causes and mechanisms underlying RPL, which will guide clinical management of such conditions and improve women's health.

RÉSUMÉ

Environ 10 à 20 % des grossesses cliniquement reconnues se soldent par une fausse couche. La survenue d'au moins deux de ces événements avant la 24e semaine de gestation est appelée perte de grossesses récurrentes et touche 1 à 5 % des couples qui tentent de concevoir un enfant et environ la moitié de ces cas restent cliniquement inexpliqués. Le séquençage de l'exome de patientes atteintes de fausses couches à répétition a révélé une mutation homozygote non-sense dans *HORMAD2* chez une patiente avec huit fausses couches récurrentes et sans enfants. HORMAD2 est une protéine essentielle pour le complexe synaptonémal. L'évaluation morphologique microscopique d'une des fausses couches de la patiente a confirmé le diagnostic. Le génotypage des microsatellites sur l'ADN disponible de deux autres fausses couches a démontré qu'elles sont triploïdes digyniques et sont la conséquence de l'échec de la méiose II maternelle. L'analyse des micropuces de polymorphismes nucléotidiques simples a révélé une anomalie supplémentaire de la méiose I, à savoir la ségrégation des deux chromosomes homologues maternels 16 et 19 dans une des fausses couches. Mes données amélioreront la compréhension actuelle des causes génétiques et mécanismes sous-jacents des fausses couches à répétition, ce qui guidera la gestion clinique de ces conditions et améliorera la santé des femmes.

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LIST OF ABBREVIATIONS

aCGH	array comparative genomic hybridization
ACMG	the American college of medical genetics and genomics
AD	autosomal dominant
AEs	axial elements
AMH	anti-müllerian hormone
ANXA5	Annexin A5
APS	antiphospholipid syndrome
AR	autosomal recessive
ART	assisted reproductive technologies
ASRM	American society for reproductive medicine
B	benign
BAF	beta allele frequency
BMI	body mass index
CADD	combined annotation dependent depletion
CCNB3	Cyclin B3
CEs	central elements
CHM	complete hydatidiform moles
CO	crossover
comp het	compound heterozygous
DFI	DNA fragmentation index
DMRs	differentially methylated regions
DNA	deoxyribonucleic acid

DNAm	DNA methylation
DSBs	double-stranded breaks
ESHRE	European society of human reproduction and embryology
FFPE	formalin-fixed paraffin-embedded
FISH	florescence *in situ* hybridization
FSH	follicle-stimulating hormone
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotropin
het	heterozygous
HM	hydatidiform moles
hom	homozygous
ICSI	intracytoplasmic sperm injection
IHC	immunohistochemistry
IUI	intrauterine insemination
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
KHDC3L	KH domain containing 3 like
LB	likely benign
LEs	lateral elements
LH	luteinizing hormone
LoF	loss of function
LOH	loss of heterozygosity
LP	likely pathogenic

LPD	luteal phase defect
MC	miscarriage
MI	Meiosis I
MII	Meiosis II
MLID	multilocus imprinting disturbance
MRI	magnetic resonance imaging
N.	number of individuals
NLRP2	NLR family pyrin domain containing 2
NLRP5	NLR family pyrin domain containing 5
NLRP7	NLR family pyrin domain containing 7
NOA	non-obstructive azoospermia
OOEP	oocyte expressed protein
P	pathogenic
PADI6	peptidyl arginine deiminase 6
PB1	first polar body
PB2	second polar body
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PGT	preimplantation genetic testing
PHM	partial hydatidiform moles
POCs	products of conception
POI	primary ovarian insufficiency
PSSC	premature separation of sister chromatids

RCOG	royal college of obstetrics and gynaecologists
RHM	recurrent hydatidiform moles
RI-MUHC	Research Institute of the McGill University Health Centre
RM	recurrent miscarriage
RNA	ribonucleic acid
ROS	reactive oxygen species
RPL	recurrent pregnancy loss
SAC	spindle assembly checkpoint
SC	synaptonemal complex
SCMC	subcortical maternal complex
SNP	single nucleotide polymorphism
STR	short tandem repeat
SYCP3	synaptonemal complex protein 3
TFs	transverse filaments
TLE6	TLE family member 6
VUS	variant of unknown significance
WES	whole exome sequencing
YCM	Y-chromosome microdeletions

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FORMAT OF THE THESIS

The thesis is presented in a manuscript-based format according to the Thesis Preparation Guidelines provided by the McGill Graduate and Postdoctoral studies website. The studies described here were performed under the supervision of Prof. Rima Slim. This thesis is comprised of five chapters, of which one chapter is in the form of a published manuscript. Chapter 1 is a general introduction which recapitulates the various aspects of the pathology of recurrent pregnancy loss and relevant background to this thesis. Chapter 2 contains a manuscript that was published in *Molecular Genetics & Genomic Medicine* in 2024 (PMID: 38400599) (Liang et al., 2024). Chapter 3 is a general discussion of the findings from this thesis. Chapter 4 comprises the conclusion and possible future directions of this project. The references are provided in Chapter 5. The appendices at the end of the thesis contain a list of internet resources used and one additional manuscript that was published in *the Journal of Assisted Reproduction and Genetics* in 2024 (PMID: 38277113) (Yalcin et al., 2024).

CONTRIBUTION OF AUTHORS

This thesis is based on the following original manuscripts.

Chapter 2

A homozygous stop codon in *HORMAD2* in a patient with recurrent digynic triploid miscarriage

Manqi Liang, Beena Suresh, Eric Bareke, Sanaa Choufani, Sujatha Jagadeesh, Rosanna Weksberg, Jacek Majewski, Rima Slim

Molecular Genetics & Genomic Medicine, 2024 Feb 23.

(PMID: 38400599)

Author contributions: I performed variant filtering, variant pathogenicity prediction, POC evaluation using various methods, and data analysis. Beena Suresh and Sujatha Jagadeesh referred the patient and gathered clinical information and materials from the conceptions. Eric Bareke and Jacek Majewski processed raw data of exome sequencing and conducted variant calls. Sanaa Choufani and Rosanna Weksberg performed the methylation microarray and guided its analysis. Rima Slim planned and provided funding for this project, supervised and guided data analysis. Rima Slim and I wrote the manuscript. All authors read and commented on the manuscript.

Appendix II

A report of two homozygous *TERB1* protein-truncating variants in two unrelated women with primary infertility

Zeynep Yalcin, **Manqi Liang**, Ibrahim M. Abdelrazek, Corinna Friedrich, Eric Bareke, Amira Nabil, Frank Tüttelmann, Jacek Majewski, Ebtesam Abdalla, Seang-Lin Tan, Rima Slim

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Author contributions: I extracted DNA from the patient and family member, submitted the sample for exome sequencing, analyzed and evaluated the pathogenicity of variants. Zeynep Yalcin validated and segregated the variant, evaluated the pathogenicity of variants, reached out to Dr. Frank Tüttelmann and Dr. Corinna Friedrich to get case 2. Ibrahim Abdelrazek, Amira Nabil, and Ebtesam Abdalla referred Case 1 and provided clinical information. Seang-Lin Tan is a partner on a MITACS project. Eric Bareke and Jacek Majewski processed raw data of exome sequencing and conducted variant calls. Rima Slim planned and provided funding for this project, supervised and guided data analysis. Zeynep Yalcin and Rima Slim wrote the manuscript.

1 GENERAL INTRODUCTION

1.1 Definitions and epidemiology of human recurrent reproductive failure

Approximately half of clinically recognized pregnancies fail to give live births (Rai & Regan, 2006; Tise & Byers, 2021). Adverse pregnancy outcomes may occur anytime from conception till delivery. Human reproductive failure manifests in a variety of forms including infertility, hydatidiform mole (HM), and pregnancy loss (Bender Atik et al., 2018; Cao et al., 2022; Choudhury & Knapp, 2001; Rai & Regan, 2006; Tomkiewicz & Darmochwał-Kolarz, 2023). The reoccurrence of such traumatic event brings physical, psychological, social, and financial strain to the patients and their families (Bender Atik et al., 2023; Eliwa et al., 2024; Turesheva et al., 2023).

Infertility is defined by the absence of clinical pregnancy after one year of regular unprotected sexual intercourse (Babakhanzadeh et al., 2020; ESHRE Capri Workshop Group, 2017; Vander Borght & Wyns, 2018). It is estimated to affect 8-15% of reproductive-age couples, and can be further classified into female infertility and male infertility. In females, primary infertility describes women who have no previous clinically recognized pregnancies, while secondary infertility describes women who become infertile after one or more previously diagnosed pregnancies (Sormunen et al., 2018; Vander Borght & Wyns, 2018).

HM, also called molar pregnancy, is an abnormal pregnancy characterized by abnormal trophoblastic proliferation (overgrowth of the placenta) and abnormal or no foetal development (Fisher & Maher, 2021; Slim et al., 2022). It can be classified as complete (CHM) or partial HM (PHM) based on microscopic morphological evaluation by histopathology, immunohistochemistry (IHC) using an antibody against p57 protein, and genotyping. Having

experienced a single HM increases the risk of a woman of having a second HM to 1-4%, and to ~13% after two or more HMs, and is referred as recurrent HM (RHM). It is not uncommon for PHMs and early RPL to be misdiagnosed, and their misdiagnosis most often depends on the evaluation methods (morphology, p57 IHC, and genotyping) and the experience of the pathologist.

Pregnancy loss is defined by the European Society of Human Reproduction and Embryology (ESHRE) guidelines (Bender Atik et al., 2018) as the spontaneous demise of confirmed pregnancies (by a positive beta-human chorionic gonadotropin (hCG) level) any time from the time of conception until 24 weeks of gestation (Banjar et al., 2023; Eliwa et al., 2024; Turesheva et al., 2023). Recurrent pregnancy loss (RPL) is the occurrence of at least two such events and affects 1-5% of couples attempting to conceive. Several other international societies of reproduction and gynecology such as the American Society for Reproductive Medicine (ASRM) and the Royal College of Obstetrics and Gynaecologists (RCOG) define RPL as having two to three consecutive or non-consecutive pregnancy losses before 14-24 weeks gestation (Eliwa et al., 2024; Practice Committee of the American Society for Reproductive Medicine, 2012; Regan et al., 2023; Turesheva et al., 2023). RPL does not include ectopic pregnancies and molar pregnancies (Bender Atik et al., 2018; Dimitriadis et al., 2020). The term "recurrent miscarriage" (RM) is reserved for cases where all pregnancy losses have been confirmed by ultrasonography or histology as intrauterine conceptions (Dimitriadis et al., 2020; Turesheva et al., 2023). Having two previous pregnancy losses raises the risk of having a subsequent miscarriage to 30%, and 33% after three losses (Ford & Schust, 2009). Early miscarriages that occur during the first trimester are more frequent in women with recurrent miscarriages compared to late miscarriages that occurs during the second trimester (Garrido-Gimenez & Alijotas-Reig, 2015), which

suggests that early miscarriages are more likely to be caused by an underlying maternal germline defect. RPL is further classified into primary and secondary, depending on the presence or absence of a previous pregnancy beyond 24 weeks of gestation (Garrido-Gimenez & Alijotas-Reig, 2015; Sultana et al., 2020).

Although infertility, RHM, and RM are different clinical entities, they share some causative genetic and non-genetic factors, and can occur in the same patient (Qian et al., 2018). Previous members from our laboratory demonstrated that patients with an HM and miscarriages have a higher risk of having aneuploid miscarriages compared to patients with sporadic or recurrent miscarriages without HM (Khawajkie et al., 2020; Qian et al., 2018). This thesis focuses primarily on the maternal genetic causes of RM.

1.2 Sporadic pregnancy loss

1.2.1 Symptoms

It is estimated that 9-20% of clinically recognized pregnancies result in miscarriages, and this number increases to 30-50% when preclinical pregnancies are included (Dimitriadis et al., 2020; Rajcan-Separovic, 2020). Symptoms associated with a higher incidence of early pregnancy loss includes lower abdominal cramping and vaginal bleeding, with more severe bleeding associated with higher pregnancy loss rates (Sapra et al., 2016). However, first-trimester vaginal bleeding is not limited to miscarriages, and occurs in ~20% of women before 20 weeks of gestation (Griebel et al., 2005). Other differential diagnoses include vaginal trauma, vaginal or cervical infection, subchorionic hemorrhage, ectopic pregnancy, and HMs. Vaginal bleeding may also happen during a viable pregnancy. Some early pregnancy losses and accompanying bleeding may occur

so early even before the pregnancy is recognized and be incorrectly classified as heavy menses, thus leading to an underestimation of the incidence of these events. On the other hand, vomiting was found negatively associated with pregnancy loss rates (Sapra et al., 2016), but is positively associated with HM and is attributed to the high level of hCG (Niebyl, 2010).

1.2.2 Diagnosis

Common reliable tools clinically used for the diagnosis of early spontaneous pregnancy loss are ultrasonography and biochemical markers such as hCG (Allison et al., 2011; Gnoth & Johnson, 2014; Griebel et al., 2005; Jurkovic et al., 2013). Transvaginal ultrasonography is used for diagnosing of intrauterine pregnancy failure by the absence of an embryo within a gestational sac with mean sac diameter greater than 25 mm or the absence of cardiac activity in a visible embryo with crown-rump length greater than 7 mm (Doubilet et al., 2014; Murugan et al., 2020). The presence of fetal cardiac activity on ultrasound examination reduces the risk of spontaneous loss to 3-6%. A follow-up by ultrasonography after approximately two weeks is often recommended to avoid misdiagnosis.

Biochemical markers are useful for confirming early pregnancy loss diagnosis for patients with non-definitive ultrasound results. hCG is produced by the syncytiotrophoblasts after implantation and is required for the maintenance of pregnancy (Gnoth & Johnson, 2014; Handschuh et al., 2007). This marker is commonly used for the detection of pregnancy because it can be detected in the maternal blood and urine during early pregnancy. hCG concentration in the maternal serum normally doubles every 1.5 days from detection up to 35 days of gestation, then every 2-3 days from 35-42 days of gestation, and reaches its highest level by week 10 then starts to decrease. Slower doubling time and decline in hCG levels are associated with spontaneous

abortion and ectopic pregnancy, although there is considerable overlap of the hCG levels in viable pregnancies, nonviable intrauterine pregnancies, and ectopic pregnancies (Deutchman et al., 2009; Doubilet et al., 2014). Other tests, including speculum and digital pelvic examination, are also used for the clinical evaluation in combination with physical examination and patients' medical and family history analysis (Allison et al., 2011).

Early pregnancy loss is subcategorized as complete, incomplete, and delayed pregnancy loss (Allison et al., 2011). A complete pregnancy loss is indicated by the complete passage of products of conception (POCs) without the need for medical intervention, and closed cervix. Incomplete pregnancy loss describes the partial passage of a POC with some retained products, and either open or closed cervix. Delayed pregnancy loss (including blighted ovum, anembryonic pregnancies, and missed abortions) is characterized by closed cervix and lack of tissue passage (Allison et al., 2011).

Tissues of POCs obtained from patients who elected for uterine curettage are routinely examined by histology in North America and the UK to confirm ultrasound diagnosis of intrauterine pregnancy and to exclude gestational trophoblastic disease (Jindal et al., 2007; Jurkovic et al., 2013). The histological evaluation is mainly based on morphological features such as irregularity of the villous contour, presence of excessive trophoblastic development, the appearance of hydropic changes in villous stroma, the absence of fetal vasculature and fibrin deposits in the intervillous space (Jauniaux & Burton, 2005). Morphological evaluation of placental tissues is commonly applied to molar pregnancies for diagnosis and for distinguishing PHM from CHM (Jauniaux & Burton, 2005; Novais Nogueira Cardoso et al., 2021). DNA extraction from formalin-fixed paraffin-embedded (FFPE) POCs and microsatellite analysis methods are becoming more commonly used and accessible (Khawajkie et al., 2019). A combination of

ultrasound examination and histopathological and genetic evaluations will allow a more comprehensive diagnosis and a better understanding of the etiologies of early pregnancy loss (Lathi et al., 2011).

For non-molar pregnancies, there exists some controversy about the usefulness of placental evaluation by morphology in determining the etiology of early pregnancy loss and understanding adverse outcomes in pregnancy complications (Jauniaux & Burton, 2005; Redline et al., 2023). Indeed, morphological placental evaluation does not allow reliable differentiation between sporadic and recurrent pregnancy loss, and in most cases does not add to the determination of the etiology of first trimester loss or clinical management (Jindal et al., 2007; Lathi et al., 2011). The detection of subtle histological features requires experienced pathologists, and the procedure raises the time and cost of routine surgery (Jauniaux & Burton, 2005; Jindal et al., 2007; Novais Nogueira Cardoso et al., 2021). Limit in the amount of material available adds difficulties to such analysis. Nevertheless, several groups emphasize the importance and benefit of having a comprehensive histopathological evaluation for the following clinical utilities: (1) confirmation of intrauterine pregnancy; (2) exclusion of gestational trophoblastic disease; (3) identification of unsuspected diseases affecting the mother or the embryo; (4) identification of conditions with an increases risk of recurrence such as chronic intervillositis, villitis, massive perivillous fibrin deposition/maternal floor infarction, maternal malperfusion, and plasma cell deciduitis; (5) understanding of adverse outcomes and guiding the management of future pregnancies (Lathi et al., 2011; Novais Nogueira Cardoso et al., 2021; Redline et al., 2023).

1.2.3 Management

Dilatation and curettage is traditionally used for the evacuation of POC from the uterus (Griebel et al., 2005; Jurkovic et al., 2013). More recently, three main types of management became available depending on the patient's condition and personal choice, namely expectant, medical, and surgical management (Allison et al., 2011; Griebel et al., 2005; Jurkovic et al., 2013). Expectant management is more commonly opted by patients because it involves the least medical intervention. For women with complete pregnancy loss confirmed by ultrasound and evaluation of the passaged POC, no further action is required. In other cases, patients are usually advised to wait for one to two weeks for the spontaneous completion of the pregnancy loss (only when signs of infection and possibility of ectopic pregnancy are ruled out), but this process may sometime take up to one month to complete. The success rate of this method is dependent on whether the pregnancy loss was incomplete or delayed, with 55-96% of women with incomplete pregnancy loss and 16-76% of women with delayed pregnancy loss achieved success with expectant management and did not require medical intervention.

Medical management most commonly involves a prostaglandin analogue misoprostol, and sometimes in combination with an anti-progesterone mifepristone to increase expulsion rates. This drug can be administered orally, vaginally, sublingually, or rectally in single or multiple doses. Vaginal bleeding is expected to start within hours of administration of misoprostol and last up to three weeks. Common side effects includes gastrointestinal distress (nausea, vomiting, diarrhea, and abdominal pain), fever, and chills. The success rate of this method is 66-99% for women with incomplete or delayed pregnancy loss in the first trimester (2.77 higher success rate compared to expectant management), and varies depending on drug dosage, route of administration, and the time allowed for POC to be passed.

Surgical management is the primary treatment option offered to women with severe bleeding or pain, hemodynamically unstable, signs of infection, provisional diagnosis of gestational trophoblastic disease, or failed conservative management (Allison et al., 2011; Jurkovic et al., 2013). The conventional procedure involves electric vacuum aspiration performed in the operating room, with a success rate of 97-98%. Manual vacuum aspiration can be carried out in the outpatient setting, with a success rate of 95-98%. This method has more recently been applied due to its multiple benefits such as reduced anesthetic risks, expenses, time of procedure, blood loss and postoperative pain. Both approaches have similar risks to surgical complications including uterine perforation, cervical laceration, hemorrhage, and intrauterine synechiae, which affects 2-8% of women undergoing surgery. The use of ultrasound guidance and antibiotic prophylaxis has been reported to reduce operative complications. Overall, surgical management has a 1.44-fold higher success rate than medication management employing a prostaglandin analogue, and with reduced nausea and vomiting.

1.3 Etiologies of recurrent pregnancy loss

RPL is a highly heterogeneous entity and has multiple etiologies (Eliwa et al., 2024; Turesheva et al., 2023). Limitations in comprehensive clinical investigation and laboratory evaluation, and varied guidelines and definitions add to the challenges in the homogenization of this condition and the standardization of the evaluation of affected couples. It is estimated that 50% of cases remain idiopathic (Cao et al., 2022; Eliwa et al., 2024; Turesheva et al., 2023). Advanced maternal age is associated with risks of sporadic pregnancy loss and embryonic aneuploidy, as increase in women's age is accompanied by diminished ovarian reserve and/or reduced oocyte quality (Dimitriadis et al., 2020; Garrido-Gimenez & Alijotas-Reig, 2015; Herbert et al., 2015;

Saravelos & Regan, 2014). The risk of pregnancy loss is the lowest, around 9.8%, in women aged at 25-29 years, and rises to 33.2% at 40-44 years, and even greater than 50% for women older than 45 years. Likewise, the proportion of aneuploid embryos is around 25-35% in women aged <35 years, and rises to 55-85% in women aged 40-45 years. Indeed, studies on human and mouse oocytes demonstrated age-related decrease in meiotic machineries such as cohesins and consequently increase in meiotic errors (Chiang et al., 2012; Tsutsumi et al., 2014).

Widely recognized etiologies of RPL includes chromosomal abnormalities, endocrine abnormalities, uterine anatomical abnormalities, thrombophilic and immunological disorders, environmental and lifestyle factors, and male factors (Figure 1.1) (Bender Atik et al., 2023; Dimitriadis et al., 2020; Eliwa et al., 2024; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Stephenson & Kutteh, 2007; Sultana et al., 2020). It is therefore important to have a complete evaluation of the medical and family history of the patients for the investigation of potential causes of RPL.

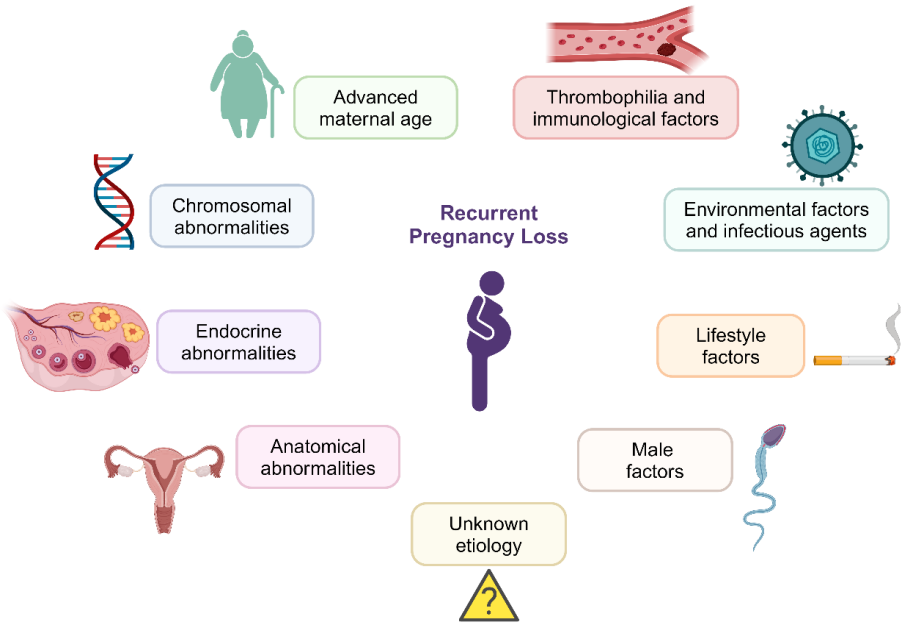

Figure 1.1 Etiologies of recurrent pregnancy loss

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1.3.1 Chromosomal abnormalities

Chromosomal abnormalities in the products of conception are most commonly found in early pregnancy loss (Garrido-Gimenez & Alijotas-Reig, 2015; Rai & Regan, 2006; Sultana et al., 2020), and they occur in approximately half of the sporadic and recurrent first-trimester pregnancy losses (Finley et al., 2022; Sahoo et al., 2017). These chromosomal abnormalities can be present in either male or female gametes or arise *de novo* in the embryo (Dimitriadis et al., 2020; Garrido-Gimenez & Alijotas-Reig, 2015).

Meiotic non-disjunction during gamete formation, mitotic error during zygote cell division, balanced reciprocal translocation, Robertsonian translocation, inversion, insertion, duplication or deletion in parental chromosomes are the common causes of embryonic aneuploidy, chromosomal structural abnormalities, or mosaicism (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Sahoo et al., 2017; Sultana et al., 2020; Yatsenko et al., 2021). Chromosomal abnormalities in the embryo can interfere with implantation, cell proliferation, placental growth or other pathways for embryonic development, which may result in an alteration in placental morphology, histology, hormones and protein secretions and increase in apoptosis (Sultana et al., 2020).

Metaphase karyotyping is primarily used for assessing numerical and structural abnormalities (Lathi et al., 2011; van den Berg et al., 2012). Other commonly used methods for the same purpose include SNP microarrays, array comparative genomic hybridization (aCGH), and florescence *in situ* hybridization (FISH) with centromeric probes (Lathi et al., 2011; J. S. Lee et

al., 2018; Sahoo et al., 2017; van den Berg et al., 2012; Webster & Schuh, 2017; Yatsenko et al., 2021; Yurov et al., 1996). Each of these methods has their own sample requirements, limitations, and advantages (in detection of small structural abnormalities, detection of balanced structural chromosome rearrangements, or the ability to exclude maternal cell contamination) (Table 1.1). A combination of methods (depending on sample availability) may provide a more comprehensive evaluation of POCs.

Table 1.1 Common techniques used for detection of chromosomal abnormalities

Technique	Karyotype	SNP microarray	aCGH	FISH*
	Detection			
Polyploidy	Y	Y	N	Y
Aneuploidy	Y	Y	Y	Y/N
Unbalanced translocation	Y	Y	Y	Y/N
Balanced translocation/Inversion	Y	N	N	Y/N
Duplication/Deletion	Y	Y	Y	Y/N
Spatial information of duplication	Y	N	N	Y
Small copy number variant	N	Y	Y	N
Mosaicism	Y	Y/N	Y/N**	Y/N
Copy number neutral LOH	N	Y	N	N
Maternal cell contamination	N	Y	N	N
	Accepted sample type			
Fresh tissue	Y	Y	Y	Y
Culturing required	Y	N	N	Y/N
FFPE tissue	N	Y	Y	Y

Abbreviations: SNP, single nucleotide polymorphism; aCGH, array comparative genomic hybridization; FISH, florescence *in situ* hybridization; LOH, Loss of heterozygosity; FFPE, formalin-fixed paraffin-embedded; Y, Yes; N, No. * Depending on the selected probes, and only known genetic abnormalities can be detected. ** aCGH cannot reliably detect low-level mosaicism below 30% (Van den Veyver & Beaudet, 2006). Table was adapted from Bishop (Bishop, 2010).

Culture based karyotyping is routinely used by cytogeneticists for the detection of both balanced and unbalanced structural rearrangements, however it has a limited resolution of 5-10 Mb, requires live cell culturing, and may lead to maternal cell contamination (Lathi et al., 2011; J. S. Lee et al., 2018; Sahoo et al., 2017). SNP microarray, aCGH, and FISH can be performed on either fresh or FFPE tissues. SNP microarray allows exclusion of maternal contamination and has a higher resolution than conventional culture-based metaphase karyotyping (Eliwa et al., 2024; Lathi et al., 2011). aCGH and SNP microarray both cover the entire genome with a resolution that depends on the probe density, but cannot detect balanced structural chromosome rearrangements nor provide information about the spatial organization of the genomic rearrangements (Lathi et al., 2011; Oostlander et al., 2004; Szuhai & Vermeer, 2015; Van den Veyver & Beaudet, 2006). Moreover, aCGH cannot detect maternal cell contamination, triploidy, nor low frequency mosaicism. FISH can be applied to both metaphase chromosomes and interphase nuclei to visualize structural and numerical chromosomal abnormalities (Bishop, 2010; Cui et al., 2016). Since FISH does not require dividing cells, it can also be used for prenatal aneuploidy testing of amniotic fluid (for a few selected chromosomes). A major limitation of FISH is that only known genetic abnormalities can be detected when using a limited number of probes.

Interphase FISH performed on 855 FFPE tissues from first trimester spontaneous abortions using eight probes detected autosomal trisomy in 258 samples (30%), polyploidy in 100 samples (12%), X chromosome monosomy in 60 samples (7%), and other monosomy/trisomy in 12 samples (1%) (Russo et al., 2016). Among approximately 4000 sporadic/recurrent pregnancy loss samples evaluated by SNP-based array or aCGH for clinically significant chromosomal abnormalities, single trisomy is the most frequent and makes up 63.3% of the cases, with

triploidy and monosomy X accounting for 11.8% and 11.2% of the cases, respectively (Sahoo et al., 2017). Embryonic aneuploidy is more frequent in early RPL compared to late RPL after 13-16 weeks of gestation (Dimitriadis et al., 2020; Garrido-Gimenez & Alijotas-Reig, 2015). In one study, aCGH of POCs alone was able to explain 57% of 378 cases of RPL, and in combination with evaluation following ASRM guidelines (excluding parental karyotyping), over 90% of the pregnancy losses were explained (Eliwa et al., 2024).

In another study employing G-banding karyotyping, 12.1% of the analyzed samples (750 POCs from women with RPL) were found to have structural chromosome abnormalities, among these 5.1% have unbalanced reciprocal translocations (Yatsenko et al., 2021). Balanced structural translocation in one partner are found in 2-5% of couples experiencing RPL (more common in the female), which increases their risk of having an unbalanced translocation in the conception and consequently lead to miscarriage (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015). However, parental peripheral blood karyotyping cannot detect parental chromosomal abnormalities in rare cases of parental gonadal mosaicism (Yatsenko et al., 2021). Mosaicism in the POCs (including mosaic autosomal aneuploidy, mosaic sex chromosome aneuploidy, mosaic triploidy/tetraploidy, and mosaic segmental aneuploidy) were found in 4.9% and 5.5% of abnormal cases by SNP-based/CGH array and karyotyping, respectively (Sahoo et al., 2017; Yatsenko et al., 2021). Nevertheless, genetic mosaicism in the embryo does not always lead to pregnancy loss (Dimitriadis et al., 2020). Successful pregnancy may be achieved if the population of aneuploid cells are eliminated or restricted to placental cell lineage.

Chromosomal abnormalities in parental DNA may be the cause of a small proportion of pregnancy losses in couples with RPL. On the other hand, the reoccurrence of chromosomal

abnormalities in the POC could be a consequence of advanced maternal age or other oocyte developmental problems and is suggestive of a genetic cause leading to the chromosomal abnormalities.

1.3.2 Endocrine abnormalities

Endocrine dysfunction, including luteal phase defect (LPD), thyroid dysfunction, diabetes mellitus, and polycystic ovarian syndrome (PCOS), is believed to account for 15-20% of RM (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Sultana et al., 2020). LPD is a condition resulting from lack of progesterone production by the corpus luteum and endometrial maturation for normal implantation and maintenance of early pregnancy. Untreated hypothyroidism and poorly managed diabetes mellitus have been associated with RPL. PCOS is often diagnosed by irregular ovulation, anovulation, hyperandrogenism, and the presence of polycystic ovaries on ultrasonography. This condition has been associated with many pregnancy complications such as gestational diabetes and pre-eclampsia. Additionally, congenital uterine anomalies have been associated with PCOS in infertile patients (Saleh & Shawky Moiety, 2014). However, the direct mechanism of PCOS in causing RPL is unclear.

Primary ovarian insufficiency (POI) is a multifactorial condition and one of the main causes of female infertility and subfertility (De Vos et al., 2010; Rossetti et al., 2017; Verrilli, 2023). This condition is characterized by arrest in ovarian function in women before the age of 40, which is diagnosed by amenorrhea (>4 month) and elevated follicle-stimulating hormone level, and it can be caused by hormonal problems, defective oocyte/follicle, or diminished ovarian reserve (Huang et al., 2021; Ke et al., 2023; L. Zhang et al., 2017). Approximately 90 genes have been implicated in POI, many of which have functions in gonadal/follicle development, oocyte

meiosis, endocrine and metabolism, and some of them are also associated with RHM or other forms of reproductive failure (Huang et al., 2021; Ke et al., 2023). Commonalities in factors and pathways that contribute to poor quality oocytes or impaired ovarian functions may underlie the spectrum of human recurrent reproductive failure (depending on the severity of the defect in the oocyte and its impact on oocyte development, fertilization, implantation, or further embryo development) (Dean et al., 2018).

1.3.3 Uterine anatomical abnormalities

Uterine abnormalities can be congenital (arcuate, septate, unicornate, bicornate and didelphis uteri) or acquired (uterine myomas, endometrial polyps and intrauterine adhesions or Asherman syndrome), which are often diagnosed by 3D ultrasound, magnetic resonance imaging (MRI), hysteroscopy or hysterosalpingography in the clinic (Dimitriadis et al., 2020; Eliwa et al., 2024; Sultana et al., 2020). Congenital uterine abnormalities are found in 10-15% of women who experienced recurrent miscarriage, which is higher than the frequency of congenital uterine abnormalities in the general population (5.5%).These abnormalities are more commonly associated with late first trimester and second trimester miscarriages (Chan et al., 2011; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Sultana et al., 2020). Among these, septate uterus is the most commonly associated with miscarriages, and has a higher frequency in women with primary RPL compared to secondary. Several genes implicated in the female reproductive tract malformations have been identified which include *HOXA13*, *HNF1B*, *LHX1*, *WNT4*, *WNT7A*, and *WNT9B* (Yatsenko & Rajkovic, 2019).

1.3.4 Thrombophilia and immunological factors

Both hereditary and acquired thrombophilia have been found to predispose women to RPL by affecting blood coagulation and increasing the risk of venous thromboembolism (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Rai & Regan, 2006; Sultana et al., 2020). Mutations resulting in deficiencies of factor V Leiden, factor II, protein C, protein S, methylenetetrahydrofolate reductase, anti-thrombin and prothrombin are common in inherited thrombophilia.

Antiphospholipid Syndrome (APS) is an acquired thrombophilia found in 5-20% of women with recurrent miscarriage, and in 3-5% of the general population. It is considered the most important treatable cause of RPL and has been discussed both in the context of thrombophilic and immunologic disorders and association with RPL and other obstetric complications, although the mechanism is not completely understood. Antiphospholipid antibodies include lupus anticoagulant and anticardiolipin antibodies. APS manifests clinically by the presence of vascular thrombosis and/or pregnancy morbidity such as recurrent miscarriage, stillbirth, fetal growth restriction and pre-eclampsia. Laboratory criteria of APS requires positive tests (repeated at least twice and 12 weeks apart) of one of the following: medium/high levels of the anticardiolipin antibodies; positive plasma levels of lupus anticoagulant; >99th centile levels of antibodies to β2-glycoprotein I of IgG and/or IgM isotype. At least one clinical and one laboratory criterion need to be met for the diagnosis of APS. Other immunological factors, namely human leukocyte antigen, cytokines, antinuclear antibodies, and natural killer cells, were proposed to be involved in fetal rejection and pregnancy losses. In the new recommendations, however, the ESHRE did not recommend routine testing of these immunological factors as part

of the evaluation of RPL, due to the lack of conclusive evidence on their relevance (Bender Atik et al., 2023; Eliwa et al., 2024).

1.3.5 Environmental factors and infectious agents

Air pollution, organic solvents, medications, ionizing radiation, toxins, and endocrine-disrupting chemicals were also suggested to increase the risk of pregnancy loss, however, direct evidence is lacking to demonstrate their causative role (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Sultana et al., 2020).

Infectious agents such as *chlamydia*, *mycoplasma*, *ureaplasma*, *listeria*, *toxoplasma*, *rubella*, coxsackieviruses, cytomegalovirus, herpes virus and parvoviruses can cause endometrial inflammation and impair endometrial functions, and have been associated with sporadic pregnancy loss (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015). The overall contribution of infectious agents to RPL is still unclear but available reports indicate that they explain 0.5-5% of the cases (Ford & Schust, 2009).

1.3.6 Lifestyle factors

Cigarette smoking, alcohol, caffeine and cocaine consumption have been associated with sporadic miscarriages, but not with RPL (Dimitriadis et al., 2020; Ford & Schust, 2009; Garrido-Gimenez & Alijotas-Reig, 2015; Rai & Regan, 2006; Sultana et al., 2020). Additionally, the increase in sperm aneuploidy has been associated with alcohol and caffeine intake and was also reported in pesticide factory workers (Lane & Kauppi, 2019). Anxiety, depression, and psychological distress are often experienced by couples with RPL, and having these

psychological burden has been associated with immune system changes and pregnancy loss (Dimitriadis et al., 2020; Rai & Regan, 2006). Obesity (BMI >30 kg/m^2) in females has a positive correlation with RPL, and obese women are at a higher risk of having future pregnancy losses (Cavalcante et al., 2019). Although, such findings are not found in women who are overweight (BMI 25-29.9 kg/m^2). Male obesity (BMI >28-30 kg/m^2) is associated with decreased sperm concentration and motility, longer time to pregnancy, and poorer assisted reproductive outcomes (Peel et al., 2023). Moreover, obesity in rodent models increased sperm DNA damage and reactive oxygen species (ROS) formation, and reduced germ cell number in the testis. Higher sperm DNA damage increases the chance of experiencing miscarriages.

Decrease in vitamin D levels is associated with increased risk of gestational diabetes and preeclampsia, and linked to infertility and miscarriage (Bender Atik et al., 2023; Dimitriadis et al., 2020; Garrido-Gimenez & Alijotas-Reig, 2015; Sultana et al., 2020). Vitamin D deficiency has been proposed to increase the level of anti-phospholipid and anti-thyroid antibodies. In addition, the expression of vitamin D receptor was found to be lower in women with RPL than women with normal pregnancy, and having a sufficient preconception concentration of vitamin D (≥75 nmol/L) was associated with an increased chance of pregnancy and live birth (Mumford et al., 2018; Sultana et al., 2020).

1.3.7 Male factors

Although male gamete contributes half of the genetic material to the zygote, paternal factors contributing to RPL are less extensively investigated than maternal factors. Several male factors previously linked to RPL include paternal chromosomal abnormalities, advanced paternal age, environmental and lifestyle factors. In addition, sperm DNA integrity, semen quality, and Y-

chromosome microdeletions (YCM) were also underlined as important paternal factors contributing to RPL (Imam et al., 2011; Inversetti et al., 2023; Puscheck & Jeyendran, 2007; Yu & Bao, 2022).

Sperm DNA integrity is measured by DNA fragmentation index (DFI), with a threshold lower than 16.5% being good quality and higher than 30% associated with a high miscarriage rate (Imam et al., 2011; Puscheck & Jeyendran, 2007; Yifu et al., 2020). Unhealthy lifestyles such as smoking and obesity contribute to an elevated sperm DFI (Anifandis et al., 2014; Du Plessis et al., 2010). Increase in the level of sperm DNA fragmentation may lead to an increase in DNA damage of the embryo, and has been associated with unexplained RM (Cao et al., 2022). Significant differences in semen parameters such as semen volume, sperm number, sperm motility and morphology were found between men with and without RPL (Inversetti et al., 2023). YCM can lead to reduced sperm count or azoospermia (Yu & Bao, 2022). Some groups reported association between YCM and RPL (Agarwal et al., 2015; Puscheck & Jeyendran, 2007), while others did not find such association (Ghorbian et al., 2012; Piña-Aguilar et al., 2012), leaving the role of YCM in PRL controversial.

Paternal age beyond 40 was significantly associated with elevated risk of spontaneous pregnancy loss, and an even a higher risk is estimated for paternal age ≥45 years of first trimester miscarriage (du Fossé et al., 2020; Puscheck & Jeyendran, 2007; Yu & Bao, 2022). Increase in sperm DNA fragmentation, decrease in sperm quality and motility, and increase in the frequency of *de novo* genetic mutations in the offspring were also implicated in advanced paternal age. Moreover, the ability of oocytes in repairing sperm DNA fragmentation is reduced with advanced maternal age, thus, the risk of spontaneous miscarriage is more pronounced when both male and female partners attempt to reproduce at a later age. Although in human pregnancies,

meiotic errors originating from male germ cells are much lower than those originating from female germ cells (Herbert et al., 2015). With the growing recognition of the relationship between paternal factors and RPL, both male and female factors should be evaluated for couples experiencing idiopathic RPL (Inversetti et al., 2023; Yu & Bao, 2022).

1.4 Genetics of recurrent pregnancy loss

With the advancement of next-generation sequencing technologies, single gene variants in patients with recurrent reproductive failure are being increasingly reported (Cao et al., 2022; Colley et al., 2019; Robbins et al., 2019). However, less single gene variants have been associated with RPL compared to infertility and RHM, and most of the genes that have been reported to be mutated in some patients have not been repeatedly detected in other studies, which is most likely due to the high degree of genetic heterogeneity of RPL.

1.4.1 Maternal causative genes

To date, there are approximately 70 maternal RPL associated candidate genes revealed by whole exome sequencing (WES), and the majority have functions in angiogenesis, cell cycle, DNA replication/repair/methylation, immune regulation, gene expression, extracellular matrix remodeling, oocyte maturation, and embryonic development (L. Biswas et al., 2021; Cao et al., 2022; Rajcan-Separovic, 2020). Among these genes, the ones that have been repeatedly reported in different studies include Annexin A5 (*ANXA5*), Cyclin B3 (*CCNB3*), Synaptonemal Complex Protein 3 (*SYCP3*), Peptidyl Arginine Deiminase 6 (*PADI6*) and NLR Family Pyrin Domain Containing 5 (*NLRP5*).

ANXA5 codes for a placental anticoagulant protein expressed in placenta, which is important for the maintenance of blood supply to the fetus (Ang et al., 2017; Bogdanova et al., 2007; Miyamura et al., 2011; Peng et al., 2022). Several studies demonstrated that the *M2* haplotype (a combination of four SNPs) in the promoter region of *ANXA5* can reduce its expression, and is associated with a higher risk of RPL, fetal growth restriction, preeclampsia, or premature birth. Both maternal and paternal carriers can transmit the *M2* haplotype to the embryo, and increase the risk of placental thrombosis and RPL.

CCNB3 regulates cyclin-dependent kinases and serves an important role in cell cycle and oocytes maturation (C. Wang et al., 2023). A homozygous missense mutation in *CCNB3* was found in two sisters with RPL by a group in Iran. The sisters had 6 and 16 pregnancy losses, of which two were confirmed to be digynic triploid that resulted from an error at the maternal MII (Fatemi et al., 2021). Our lab also found a homozygous mutation that affects the canonical splice site of exon 12 of *CCNB3* in a patient with 16 miscarriages, and demonstrated that the only available POC from the patient is digynic triploid resulted from an error at Meiosis I (Rezaei et al., 2022).

SYCP3 encodes for an essential structural component of the synaptonemal complex (SC), which is formed between homologous chromosomes during Meiotic Prophase I and is involved in synapsis and recombination (L. Biswas et al., 2021; Bolor et al., 2009; Xie et al., 2022). Two heterozygous mutations in *SYCP3* were reported in two women who had three miscarriages each. In addition, a heterozygous frameshift mutation in *SYCP3* was reported in two infertile men with non-obstructive azoospermia (NOA), and was associated with early meiotic arrest (Miyamoto et al., 2003). A more detailed discussion of the SC is provided in Chapter 3.

Members of the subcortical maternal complex (SCMC) include NLR Family Pyrin Domain Containing 2 (*NLRP2*), *NLRP5*, NLR Family Pyrin Domain Containing 7 (*NLRP7*), TLE Family Member 6 (*TLE6*), Oocyte Expressed Protein (*OOEP*), KH Domain Containing 3 Like (*KHDC3L*), and *PADI6* (Eggermann et al., 2021; Rezaei et al., 2021). The SCMC is localized at the cortex of mammalian oocytes during maturation and plays an important role during early embryonic development and genomic imprinting. Mutations in these SCMC genes have been associated with RHM, RPL, and/or female infertility. Biallelic mutations in *NLRP7* and *KHDC3L* explains 55% and 5% of RHMs in patients, and several of these patients have also had miscarriages, which seems to depend on the severity of the variants' impact on the protein function (Rezaei et al., 2021; Slim et al., 2022). In one family of three sisters, our lab found a homozygous missense in *NLRP7*. Two sisters suffered from multiple HMs, miscarriages, and were able to achieve live birth, while the third sister was reported to have had several miscarriages, although they were not fully evaluated.

Biallelic mutations in *PADI6* and *NLRP5* have been reported in women with infertility, RHMs, and miscarriages (Rezaei et al., 2021). Our lab previously found in *PADI6*, a compound heterozygous missense mutation in a patient who had five miscarriages, one HM, and no live birth (Qian et al., 2018), and a homozygous missense in another patient with two miscarriages, four HM, and one live birth of donor embryo (Rezaei et al., 2021). A research group in Germany also reported a compound heterozygous mutation (one missense and one nonsense) in *PADI6* in a woman who suffered from different miscarriages and gave birth to two children with multilocus imprinting disturbance (MLID) (Eggermann et al., 2021).

Compound heterozygous missense mutation in *NLRP5* was reported in a woman who experienced six pregnancy losses, one HM, and two children with MLID with three unrelated

partners, and her mother had three miscarriages (Docherty et al., 2015). Another woman with a *NLRP5* compound heterozygous mutation (one missense and one nonsense) had four miscarriages, two children with MLID, and two healthy children. Sparago and colleagues (Sparago et al., 2019) reported a woman with compound heterozygous mutations (one missense and one nonsense) in *NLRP5* who had four pregnancy losses at 12-29 weeks gestation, one child with Beckwith-Wiedemann syndrome, and one child without clinical features of disease.

1.4.2 Paternal causative genes

Only 12 paternal genes have been associated with RPL, although an increasing number of investigations are being conducted as the male contribution to RPL becomes more recognized (Cao et al., 2022). The functions of the reported genes are similar to those of the maternal candidate genes, namely angiogenesis, DNA replication/methylation, mitosis, and gene expression. Genetic polymorphisms in genes such as *ANXA5*, *MTHFR*, *USP26*, *BPTF*, *MECP2*, and *SOX21* have been associated with RPL in men (Asadpor et al., 2013; Ibrahim & Johnstone, 2018; Mou et al., 2022; Tüttelmann et al., 2013; Y. Yang et al., 2016; Zheng et al., 2022). In addition, many meiotic genes were associated with male infertility, particularly NOA, including *STAG3*, *MEIOB*, *MEI1*, *SYCE1*, and *SIX6OS1* (Sudhakar et al., 2021; Y. Wang et al., 2022; Xie et al., 2022; Y. Zhang et al., 2023; Zhu et al., 2024).

1.5 Meiosis in oocytes

Meiosis is a reduction division process comprised of the DNA replication followed by two rounds of cell division, during which diploid germ cells are reduced to haploid gametes (L.

Biswas et al., 2021; França & Mendonca, 2022; Xie et al., 2022). The successful pairing and recombination between parental homologous chromosomes is crucial for the proper segregation of chromosomes in oocytes and sperm, and for the creation of genetic diversity.

Female meiosis begins in fetal ovaries in as early as 5-6 weeks in human pregnancy and spans many years (substantially longer than male meiosis) (L. Biswas et al., 2021; Charalambous et al., 2023; França & Mendonca, 2022; Lane & Kauppi, 2019; Xie et al., 2022). Following mitotic expansion of primordial germ cells and Pre-meiotic S-phase, meiosis begins with Meiotic Prophase I. Double-stranded breaks (DSBs) form following DNA replication. The meiotic cohesin complex, a ring-like protein structure installed during DNA replication, joins sister chromatids along the chromosome axis.

Meiotic Prophase I is divided into five substages, namely leptotene, zygotene, pachytene, diplotene, and diakinesis. At leptotene, chromosomes condense and attach to the nuclear envelope via their telomeres, mediated by the TERB1-TERB2-MAJIN complex (described in Appendix II - A report of two homozygous *TERB1* protein-truncating variants in two unrelated women with primary infertility). This attachment is essential to facilitate chromosome movement and homologous pairing. At zygotene, homologous chromosome synapsis begins. The SC, a proteinaceous zipper-like structure, forms a scaffold to stabilize the homologs pairing and to allow for DSBs repair. At pachytene, homologous chromosomes complete synapsis, and approximately 10% of the meiotic DSBs are repaired as crossovers (COs), creating a physical link (chiasmata) between homologs and maintain the homolog pairs as bivalents. A minimum of one crossover in each homologous chromosome pair is essential for accurate segregation at the first meiotic division (L. Biswas et al., 2021; Youds & Boulton, 2011). At diplotene, the SCs begin to dissociate (desynapsis) and homologs remain linked at chiasma (L. Biswas et al., 2021;

Lane & Kauppi, 2019; Webster & Schuh, 2017; Xie et al., 2022). Oocytes arrest at diplotene (also named dictyate stage), which can last between 13-51 years. Persistent DNA damage in the oocyte (due to failure of DSB repair) may cause apoptosis, which decreases the ovarian reserve and could lead to POI.

During the dictyate arrest, oocytes associate with surrounding somatic cells and form primordial follicles, which contain one oocyte and a single layer of granulosa cells (Charalambous et al., 2023; Fragouli & Wells, 2013; França & Mendonca, 2022). Approximately one million primordial follicles are stored in the ovaries at birth, and half of them remain by puberty as a result of follicle degeneration and reabsorption (follicular atresia). At puberty, around 20 primordial follicles begin to grow into primary follicles in a menstrual cycle (one year before ovulation), during this prolonged growth phase oocytes expand in size and granulosa cells proliferate. The oocyte and surrounding somatic cells transmit materials via gap junctions (Charalambous et al., 2023). Primary follicles transition into secondary follicles through thecal cell recruitment and proliferation (Fragouli & Wells, 2013). The follicle-stimulating hormone (FSH) binds to its receptor to promote follicle growth (França & Mendonca, 2022). One enlarged follicle (the dominant follicle) continues to develop into antral follicle, while the smaller follicles degenerate. Granulosa cells in the antral follicle are differentiated into mural granulosa cells and cumulus cells that surround the oocyte (Fragouli & Wells, 2013). In each menstrual cycle, a luteinizing hormone (LH) surge from the pituitary gland triggers ovulation and meiotic resumption in the oocyte (Charalambous et al., 2023).

Nuclear membrane begins to fragment, known as the germinal vesicle breakdown (GVBD), in the oocyte upon meiotic resumption, nucleolus disappear, meiotic spindle fibers start to form, and chromosomes begin to condense (L. Biswas et al., 2021; Charalambous et al., 2023; Lane &

Kauppi, 2019; Webster & Schuh, 2017; Xie et al., 2022). In Metaphase I, fully condensed chromosomes align at the metaphase plate, and microtubule spindles from the same spindle pole attach at the kinetochores of sister chromatids (act as a single unit). The spindles and chromosomes then migrates towards the oocyte cortex in preparation for the extrusion of the first polar body (PB1). The spindle assembly checkpoint (SAC) ensures the correct attachment of spindle microtubules to the kinetochores, and otherwise delays the progression into Anaphase I (Lane & Kauppi, 2019). Although, the SAC in oocytes is leaky, and univalents sometimes can escape its surveillance. In Anaphase I, homologs are disjoint and move towards opposite spindle poles (Uraji et al., 2018; Webster & Schuh, 2017). Chromosomes near the cortex signals actin to form the actin cap. The meiotic cohesin complex near the centromere is retained, keeping sister chromatids together.

In Telophase I, half of the chromosomes are extruded into the PB1 with the minimum cytoplasm while the other half remains in the oocyte, undergoing asymmetric cytokinesis (Uraji et al., 2018; Webster & Schuh, 2017). The actin-myosin contraction is essential in the formation of cleavage furrow. In Metaphase II, sister chromatids attach to microtubule spindles from opposite spindle poles, and align at the equator. The mature oocyte (arrested in Metaphase II), capable of fertilization and embryonic development, migrates through the fallopian tube towards the uterus. Anaphase II begins after the fertilization by a sperm, and cohesin complex at the centromere is removed and sister chromatids are separated in the zygote. The second polar body (PB2; containing half of the sister chromatids) is extruded, and maternal and paternal pronuclei are formed. The parental pronuclei join to form the first Metaphase in the zygote, which then undergoes many rounds of mitotic divisions to form a blastocyst that will hatch and implant in the uterus.

Nondisjunction in MI or MII can result in gain or loss of chromosomes (Ottolini et al., 2015; Webster & Schuh, 2017). The origin of trisomies in humans in terms of parental contribution and meiotic stage of the error varies greatly depending on the chromosome. For example, around half of the observed XXY trisomies origin from paternal MI errors, while trisomy 16 mostly, if not all, origin from maternal MI errors. In general, maternal MI errors seem to contribute the most to trisomies, and aneuploidy originating from maternal chromosomes is more frequently observed compared to that from paternal chromosomes, which may be explained by the error-prone nature of mammalian female meiosis (Nagaoka et al., 2012; Webster & Schuh, 2017). More recently, the premature separation of sister chromatids (PSSC) during the first meiotic division and separation of non-sister chromatids during the second meiotic division (termed reverse segregation) were hypothesized to result from the COs close to the centromeric regions or incorrect orientation of sister chromatids in Metaphase I (Charalambous et al., 2023; Ottolini et al., 2015; Webster & Schuh, 2017). Another mechanism is that weak cohesion at the centromeres (could be due to gradual loss of cohesins over time during maternal aging) allows PSSC, forming univalent that may interfere with chromosome alignment and segregation, and resulting in a higher gamete aneuploidy rate in women of advanced age (Charalambous et al., 2023; Hassold & Hunt, 2001). At the end, the number of chromosomes in the oocyte and PB1 may be correct, resulting in euploid oocyte. Alternatively, the unlinked non-sister chromatids can cause spindle aligning errors in Metaphase II and subsequent aneuploidy. Thus, MII segregation errors may originate from MI errors in oocytes.

Genes involved in the process of meiosis that have been associated with female infertility/subfertility include genes with roles in chromosome movement and pairing (*KASH5*, *SUN1*, *TERB1*, *TERB2*, *MAJIN*); synapsis and cohesion (*SYCP2/3*, *SYCE1*, *SIX6OS1*, *STAG3*,

TRIP13, *HORMAD1*, *HORMAD2*); double-strand break and CO (*HFM1*, *MEIOB*, *BRCA1*, *BRCA2*, *TOPBP1*, *MEI1*, *MEI4*, *REC114*, *DMC1*, *MCM8*); translation regulation (*PATL2*, *NANOS3*); meiotic cell cycle regulation (*WEE2*, *CDC20, CCNB3*); spindle formation and chromosome segregation (*TUBB8*, *CEP120*, *AURKB*, *AURKC*, *TRIP13*) (L. Biswas et al., 2021; Xie et al., 2022). Variants in these genes which have been found in women with infertility were summarized in Xie et al. and Biswas et al. (L. Biswas et al., 2021; Xie et al., 2022). In addition to the correct chromosomal segregation in meiosis, cellular and molecular regulation of the oocyte maturation within the ovary has equally important contribution to the oocyte quality (Coticchio et al., 2015). The groups of França (França & Mendonca, 2022) and Yatsenko (Yatsenko & Rajkovic, 2019) reviewed in detail the genes implicated in ovarian functions, with a focus on POI and infertility, such as genes involved in gonadogenesis (*FOXL2*, *NR5A1*, *GATA4*, *BMPR1B*); folliculogenesis (*NANO3*, *SOHLH1*, *SOHLH2*, *NOBOX*, *AMH*, *AMHR2*, *FSHR*, *INHA*); zona pellucida (*ZP1*, *ZP2*, *ZP3*); steroidogenesis (*STAR*, *GATA4*, *CYP17A1*, *CYP19A1*, *PGRMC1*); and metabolic functions (*AIRE*, *EIF4ENIF1*, *GALT*, *RCBTB1*, *TWNK*). Disruption of these protein functions often lead to abnormalities in the gametes (such as premature death or aneuploidy) and manifest in infertility (commonly as NOA and POI), subfertility, miscarriages, or fetal congenital abnormalities (L. Biswas et al., 2021; França & Mendonca, 2022; Xie et al., 2022).

1.6 Genomic imprinting

Epigenetic modifications, including DNA/RNA methylation, histone modifications, chromatin remodeling, and non-coding RNA regulation, are crucial for gene expression regulation without changing the DNA sequence, and DNA methylation has been most extensively studied among

them (Elhamamsy, 2017; Zhou et al., 2021). The methylation dynamics from the gamete to early embryonic stages is periodic and highly regulated. Abnormalities in DNA methylation may silence key genes for normal embryonic development or induce the expression of genes that should be inactive, and potentially lead to early pregnancy loss or pediatric diseases.

Genomic imprinting is a phenomenon that silences a restricted number of alleles via DNA methylation depending on their parental origin, and is tissue and stage-specific during development (Bourque et al., 2011; Fatemi et al., 2021; Slim et al., 2022; Zhou et al., 2021). Maternal *de novo* DNA methylation begins during dictyate stage in Prophase I and continues until the oocyte is fully grown (Li & Sasaki, 2011; Smallwood & Kelsey, 2012). Imprinting disorders are recognized to have important roles in embryonic development and placental function (e.g. CHM and placental mesenchymal dysplasia), which are caused by loss of function (LoF) variants in genes that belong to the SCMC (Bourque et al., 2011; Slim et al., 2022; Zhou et al., 2021). Quantification of DNA methylation at differentially methylated regions (DMRs) via pyrosequencing assays can be used for the diagnosis of epigenetic errors at various imprinting disorders, and to distinguish digynic triploidy (extra haploid set from mother) from diandric triploidy (extra haploid set from father) and healthy pregnancies (Bourque et al., 2011). For example, the methylation assay at the maternally methylated locus *SGCE* shows a clean separation between the groups mentioned above and a linear relationship between the methylation level and the relative maternal contribution to the genome. The mean methylation value for digynic triploid conception is around 62%, while it is 49% for healthy diploid control and 34% for diandric triploid, respectively.

1.7 Management of recurrent pregnancy loss

ESHRE (Bender Atik et al., 2023) and other groups provided detailed recommendations for the evaluation and treatment of couples experiencing RPL (Cao et al., 2022; Dimitriadis et al., 2020; El Hachem et al., 2017; Garrido-Gimenez & Alijotas-Reig, 2015; Homer, 2019; Rai & Regan, 2006). Genetic counselling is recommended for these couples even if the counselling does not always provide useful information. Preimplantation genetic testing (PGT) of the embryo is sometimes used during *in vitro* fertilization (IVF) to identify monogenic defects, aneuploidy, or structural rearrangements for couples with identified chromosomal abnormalities or unexplained RPL. However, evidence that shows PGT can improve live birth rate compared to expectant management is lacking, and further randomized controlled trials are needed (Bender Atik et al., 2023; Homer, 2019). Murugappan et al. (Murugappan et al., 2015) demonstrated that IVF-PGT by aCGH 24-chromosome screening was not cost-effective in improving birth outcome for couples with unexplained RPL, with a live-birth rate of 53% compared to 67% in the group using expectant management. Nevertheless, expectant management group had a higher clinical miscarriage rate of 24% and only 7% for IVF-PGT group. The observed lower live birth rate may be partially attributed to the higher maternal age in the IVF-PGT group, and thus a reduced oocyte quality in addition to the chromosomal abnormalities. It is also possible that some aneuploid cells remain in the extraembryonic tissues and do not contribute to the inner cell mass, and may achieve successful live birth. In addition, genome analysis of polar bodies can also be used for diagnosing aneuploidy in the egg (Webster & Schuh, 2017).

Patients who had a septate uterus and were treated by hysteroscopic septum resection had a reduced risk of pregnancy loss (Bender Atik et al., 2023). One study reported that patients who went through successful hysteroscopic septum resection had ~85% live birth rate and ~75% term

delivery rate (Grimbizis et al., 2001). For patients with cervical insufficiency, prophylactic cervical cerclage is recommended (Cao et al., 2022).

Levothyroxine treatment and vitamin D supplementation are recommended for women with overt hypothyroidism and women with diagnosed vitamin D deficiency, respectively (Bender Atik et al., 2023; Dimitriadis et al., 2020). Prophylactic low-molecular-weight heparin in combination with low-dose aspirin are recommended for women with APS and RPL (Bender Atik et al., 2023; Dimitriadis et al., 2020; Garrido-Gimenez & Alijotas-Reig, 2015; Rai & Regan, 2006). Lifestyle modifications such as maintaining a normal BMI (20-30 kg/m^2 for Caucasian population), having a normal exercise pattern, reducing stress and having psychosocial support, cessation of smoking, limiting alcohol consumption, and avoiding drug use and environmental hazards for both partners may improve pregnancy outcomes. Evidence is lacking for treatment of RPL of paternal etiology, although antioxidants were proposed as a possibility for reducing sperm DNA fragmentation rates (Puscheck & Jeyendran, 2007).

The prognosis of subsequent pregnancies for couples with RPL of unknown etiology is largely dependent on the cause of pregnancy losses, the couple's age and reproductive history, and family history, but is overall encouraging even without therapeutic intervention (Dimitriadis et al., 2020; Ford & Schust, 2009; Rai & Regan, 2006). Assisted reproductive technologies (ART) such as intrauterine insemination (IUI), IVF, intracytoplasmic sperm injection (ICSI), and oocyte/sperm donation with PGT may be beneficial for couples with chromosomal abnormalities or other problems in their gametes (Smeenk et al., 2023).

In conclusion, given the complexity of the etiology of RPL, a thorough evaluation for couples experiencing two or more pregnancy losses is warranted to identify the cause and to guide in the management and prognosis of this condition.

1.8 Rationale and objectives

As RPL is a highly heterogeneous entity and many of its causes remain unexplained, we hypothesized that there are some unidentified maternal genetic causes responsible for RPL under the recessive model in our cohort of patients that we planned to identify and to characterize the mechanisms of their occurrence.

Objective 1. Whole exome sequencing of the female patients, variant filtering, prioritization, validation, and segregation.

Objective 2. Elucidation of the mechanism leading to the condition by characterizing available POCs.

PREFACE TO CHAPTER 2

The highly heterogeneous etiologies of RPL complicate the search for its causative genes. The manuscript in Chapter 2 describes the genetic analysis of a couple with eight recurrent miscarriages of unexplained clinical etiology. We analyzed the available POCs using histopathology, STR genotyping, SNP microarray, and methylation analysis. These analyses revealed that two POCs are triploid digynic due to maternal MII failure. Additionally, MI abnormalities of two chromosomes were found in one POC. Through WES, we identified variants in two candidate genes with roles in female reproduction, a missense variant in *EIF4ENIF1* and a stop gain variant in *HORMAD2*. Evaluation of the function of these two genes, with consideration of the couples' clinical presentation and family history, led to the conclusion that *HORMAD2* is the most favorable causative gene.

2 A HOMOZYGOUS STOP CODON IN *HORMAD2* IN A PATIENT WITH RECURRENT DIGYNIC TRIPLOID MISCARRIAGE

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2.1 Abstract

Background

Recurrent miscarriage (RM), affects 1 to 5% of couples trying to conceive. Despite extensive clinical and laboratory testing, half of the RM cases remain unexplained. We report the genetic analysis of a couple with eight miscarriages and the search for their potential genetic etiology.

Methods

Short tandem repeat (STR) markers, single nucleotide polymorphic (SNP) microarray, and human DNA methylation microarray were used to analyze the genotypes of two miscarriages. Exomes sequencing was performed on DNA from the two partners and identified variants were validated by Sanger sequencing.

Results

STR marker genotyping demonstrated that the two available miscarriages are triploid digynic and resulted from the failure of Meiosis II. SNP microarray analysis revealed an additional Meiosis I abnormality that is the segregation of the two maternal homologous chromosomes in one triploid miscarriage. Whole-exome sequencing on DNA from the two partners identified candidate variants only in the female partner in two genes with roles in female reproduction, a missense in *EIF4ENIF1* (OMIM 607445) and a stop gain in *HORMAD2* (OMIM 618842). EIF4ENIF1 is a eukaryotic translation initiation factor 4E nuclear import factor required for the oocyte germinal vesicle breakdown, and HORMAD2 is part of the synaptonemal complex that was hypothesized to act as a checkpoint mechanism to eliminate oocytes with asynapsis during meiotic prophase I in mice.

Conclusion

While both genes may contribute to the phenotype, the Meiosis I abnormalities in the conceptions favor the causal role of *HORMAD2* in the etiology of RM in this couple. This report illustrates the importance of comprehensively analyzing the products of conception to guide the search for the genetic causation of RM.

Keywords

abnormaliy, failure of Meiosis II, *HORMAD2*, Meiosis, recurrent miscarriage, triploid digynic

2.2 Introduction

Approximately 1 to 5% of couples trying to conceive experience recurrent miscarriage (RM), which is defined by the occurrence of at least two miscarriages before 22-24 weeks of gestation (Bender Atik et al., 2018). RM can be caused by or associated with a multitude of factors that include karyotype abnormalities in either of the two partners or in their conceptions, female factors such as thrombophilia, immunological, metabolic and endocrinological, and anatomical, or male factors (Colley et al., 2019; El Hachem et al., 2017; Stephenson & Kutteh, 2007). Consequently, the evaluation of couples with RM requires comprehensive clinical and laboratory investigations of both partners and of their miscarriages that are not always available in all medical centers. Another limitation of comprehensive investigations is the coverage of the cost of these laboratory tests by public and/or private medical insurance, which is not the same in all countries and health care systems. Therefore, the heterogeneity of RM in addition to the challenges associated with the implementation of standardized, comprehensive, and systematic

evaluation of couples with RM have hampered the homogenization of this entity to facilitate its studies and reaching robust conclusions on its causative factors besides association studies on small cohorts of patients (Rull et al., 2012).

In humans, recurrent reproductive failure manifests mainly in three forms, infertility, recurrent molar pregnancy, and RM. The advent of next generation sequencing in the past 15 years has greatly advanced the genetics of infertility and recurrent molar pregnancy and led to the identification of approximately 25 of their causative genes (Biswas et al., 2021; Colley et al., 2019; Robbins et al., 2019; Sang et al., 2021). However, few genes responsible for RM have been identified.

The goal of this study was to investigate the potential genetic cause of eight consecutive first trimester miscarriages over a period of 7 years and with no live birth in a couple of Indian origin that have remained unexplained despite extensive clinical and laboratory evaluations. Here we describe the analysis of their available products of conception (POCs) and their constitutive DNA whole exome sequencing (WES).

2.3 Materials and methods

Ethical compliance

This study involves human participants and was approved by McGill University Faculty of Medicine and Health Sciences Institutional Review Board (A01-M07-03A) in 2003 and renewed yearly since. Participants gave written informed consent to participate in the study.

Histopathological characterization of one product of conception

Archived formalin-fixed paraffin-embedded (FFPE) tissues were available only from the third product of conception (POC3). This POC was sectioned, and the sections were stained with hematoxylin and eosin, and examined using bright field microscopy.

DNA extraction

Genomic DNA was extracted from peripheral blood samples from the couple and three family members using the Flexigene DNA Kit (QIAGEN) according to manufacturer's instructions. DNA extracted from freshly dissected chorionic villi were available from the seventh (POC7) and eighth POC (POC8). We attempted to genotype DNA extracted from the chorionic villi of POC3 using DNA FFPE Tissue Kit (QIAGEN). However, the DNA quality was not sufficient to obtain conclusive result.

SNP microarray

Affymetrix CytoScan HD microarray 750K was performed at The Center for Applied Genomics (Toronto, Canada) on DNA from the male and female partners and from two of their miscarriages, POC7 and POC8, from which good quality DNA extracted from freshly dissected chorionic villi was available. The genotypes were assigned based on beta allele frequency (BAF) and visualized using Chromosome Analysis Suite 4.3 (ChAS 4.3). Assignment of the genotype from BAF was performed as previously described by applying the Mendelian inheritance scheme (Usui et al., 2019; Wirtenberger et al., 2005), designating the allele contributed by the partner (AA or BB), and determining the allelic contribution of the patient at informative loci where the patient has two different alleles (AB). The BAF analysis was then manually performed using Microsoft Excel 2010.

Methylation analysis

Genome-wide DNA methylation (DNAm) profiling on DNA from POC7 and POC8 and three control POCs was performed at the Center for Applied Genomics (TCAG; SickKids Research Institute, Toronto, Ontario, Canada). DNA was sodium bisulfite converted using the EpiTect Bisulfite Kit (EpiTect PLUS Bisulfite Kit, QIAGEN, Valencia, CA) according to the manufacturer's protocol. Modified genomic DNA was then processed and analyzed on the Infinium Human MethylationEPIC BeadChip (Illumina Inc, San Diego, California) according to the manufacturer's protocol. For quality control and normalization, the raw IDAT files were converted into β-values, which represent DNAm levels as a percentage (between 0 and 1) using the minfi Bioconductor package in R. Data preprocessing included filtering out nonspecific probes, probes with detection *p*-value >0.05 in more than 25% of the samples, probes located near single nucleotide polymorphic sites (SNPs) with minor allele frequencies above 1%, and X and Y chromosome probes. A total of 91,599 probes were removed and a total of n=774,260 probes were included in the methylation analysis as previously described (Choufani et al., 2020). Standard quality control metrics in minfi were used, including median intensity QC plots, density plots, and control probe plots. All samples passed quality control and were included in the analysis.

Genetic analysis

WES was performed on blood DNA from the patient and her partner at the Centre d'expertise et de services Génome Québec (Montreal, Quebec). Agilent SureSelect Human Exome library preparation was used for exome capture and Illumina NovaSeq 6000 PE100 for sequencing with 100x average coverage. Sequences were aligned to the human genome (GRCh37/hg19). The identified variants were validated and segregated in available family members by PCR

amplification of genomic DNA and Sanger sequencing. The primers (Table S2.1) were designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Untergasser et al., 2007) and the UCSC reference genome (GRCh37/hg19; Kent et al., 2002). Variant pathogenicity prediction was performed according to ACMG guidelines (Richards et al., 2015) using VarSome (https://varsome.com/; Kopanos et al., 2019). NCBI Reference Sequence for *HORMAD2* is NM_152510.4, and for *EIF4ENIF1* is NM_019843.4. All variants detected have been submitted to the Leiden Open Variation Database (https://databases.lovd.nl/shared/individuals/00440109; patient ID 00440109). Simplex and multiplex short tandem repeat (STR) genotyping was performed on POC7 and POC8 as previously described (Khawajkie et al., 2017; Rezaei et al., 2022).

2.4 Results

Patient and clinical history

The couple was referred to our laboratory by the Department of Clinical Genetics & Genetic Counselling, Mediscan Systems, Chennai, India, to investigate a possible genetic cause that could underlie their eight RM (all around gestational age of 8-9 weeks) over 7 years and with no live birth. The two partners are not smokers and are in good health. Their body mass indexes were slightly higher than normal ranges (26.7 and 25.7 for the female and male, respectively). They both have normal karyotypes using conventional cytogenetic analysis. Semen analysis of the male partner revealed mild asthenoteratozoospermia and hypospermia, but these abnormalities were not convincing to be the cause of their eight miscarriages. The male partner has one sister who had two children, and his parents did not have miscarriages or problems

conceiving. The female partner had regular menstrual cycles. Her pelvic ultrasound revealed normal cavity, uterus size, and endometrium. In her mid-30s, her ovarian volumes were normal (8.99 cc and ovary 9.57 cc for the right and left ovaries, respectively) as well as her both adnexa. Antral follicular counts were 8 and 5 for the right and left ovaries, respectively, which is within normal range (4-24; Coelho Neto et al., 2018). The anti-Müllerian hormone (AMH), a hormone secreted by granulosa cells of maturing follicles, was measured also in her mid-30s and was normal (2.3 ng/ml). During her obstetrical evaluation, the patient tested only once positive for lupus anticoagulant. This test was repeated 3 months later and was negative. Nevertheless the patient was given prophylactic treatment and put on Ecospirin 75 mg and low-molecular-weight heparin (Enoxaparin 40mg/day) once a day from the start of the pregnancy until the date of the miscarriage for the last two miscarriages. As part of the patient work up, molecular karyotyping was performed on her seventh miscarriage (diagnosed as miscarriage by ultrasonography and microscopic histological evaluation) by quantitative fluorescent PCR analysis with primers from chromosomes 13, 18, 21, and X and Y, which demonstrated trisomy for all analyzed chromosomes suggesting a triploidy. The mother of the patient had three live births, one elective termination of pregnancy, and no history of miscarriages or primary or secondary infertility. The patient's mother had her menopause at the age of 55 years. The parents of the patient are fourth degree cousins. Also, there was no history of any form of reproductive failure in the maternal- or paternal grand-parents.

In conclusion, despite extensive testing, none of the above abnormalities in the two couples was convincing to explain their eight consecutive miscarriages with the first being when the female partner was in her mid-20s. The couple was then referred for genetic consultation with a suspicion of a maternal genetic defect, most likely because of the triploid miscarriage.

Histopathology and genotyping analysis of the miscarriages

Morphological evaluation of the third miscarriage, POC3, from which archived FFPE tissues were available, showed chorionic villi with some hydropic changes, intravillous fibrin, and absence of trophoblastic proliferation (Figure 2.1). Fetal nucleated red blood cells were not present in the chorionic villi, and the available tissues did not contain any other embryonic or extra embryonic tissues. These findings are compatible with the diagnosis of an early arrested pregnancy. DNA extracted from freshly dissected tissues from POC7 and POC8 was used to determine the parental contribution to the POC genomes, using the PowerPlex 16 HS System (Promega, Corporation, Fitchburg, WI). This assay is a multiplex microsatellite genotyping kit that amplifies alleles at 16 markers from 15 chromosomes and the X and Y amelogenin gene (Table S2.2). The analysis of the DNA of POC7 and POC8, along with parental DNA showed that at all the analyzed loci, the POCs received either two copies of the same maternal allele or two different maternal alleles along with a single paternal allele (Figure 2.1). The amelogenin marker showed that the sex-chromosome complement of POC7 was XXY and POC8 was XXX. Hence, both POC7 and POC8 were triploid digynic. This suggested a defect in chromosome segregation during maternal meiosis.

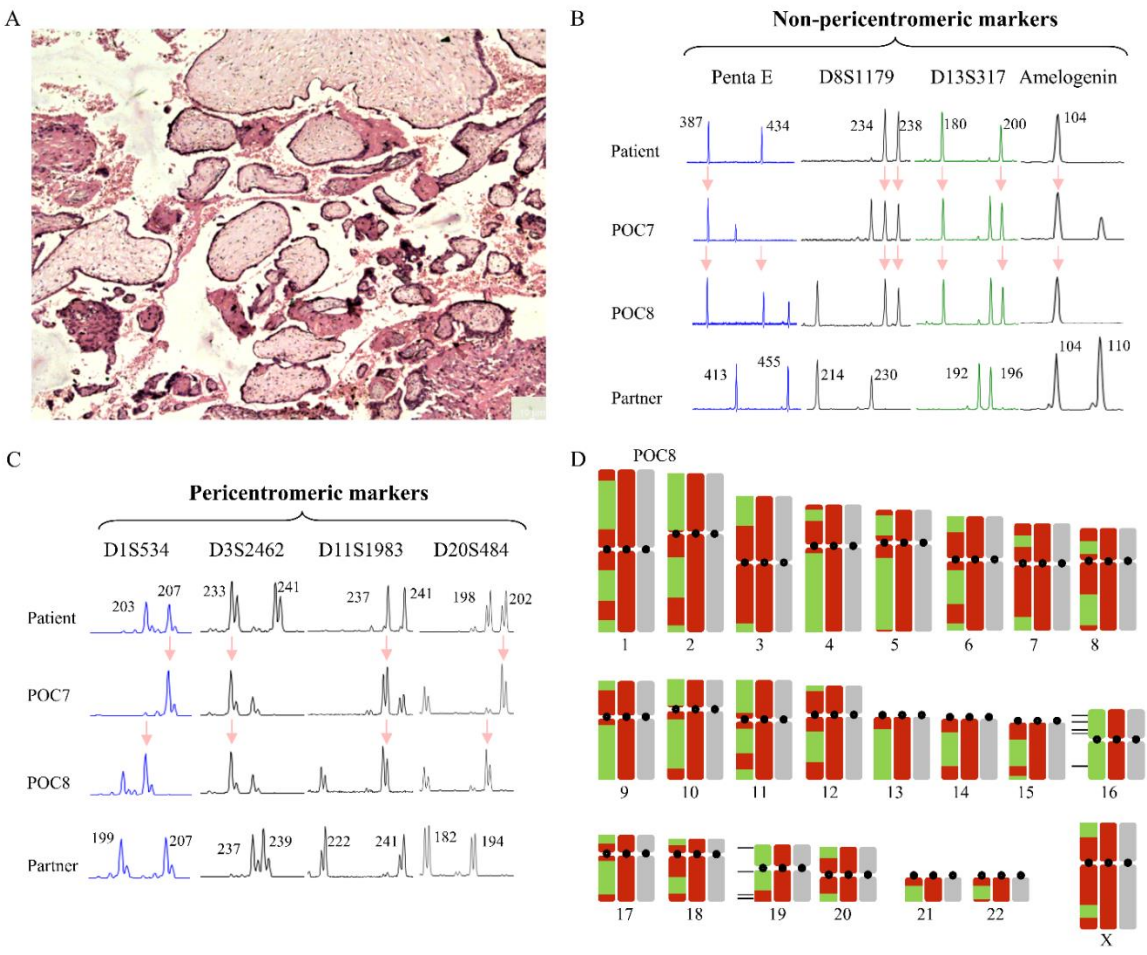

Figure 2.1 Histopathology, genotyping, and SNP microarray characterization of the products of conception (POCs)

(a) Microphotograph of POC3 showing chorionic villi with abnormal morphology. (b) Microsatellite genotyping results of POC7 and POC8 demonstrating their triploid digynic genomes. (c) Genotyping POC7 and POC8 at four pericentromeric markers showing that the triploidies resulted from the failure of meiosis II. (d) Meiotic map of POC8. Grey boxes denote chromosome inherited from the partner. Red loci denote positions where the patient's heterozygosity was reduced to homozygosity in the POC. Green loci denote positions where the patient's heterozygosity was retained in the POC. Breakpoints where red and green switches

denote sites of cross overs (cross over sites were marked on one chromosome for simplicity but they could be on either maternal chromosomes). On chromosome 16 and 19 black horizontal lines represent the positions of STR markers used to confirm the SNP microarray data. The markers used are D16S678, D16S764, D16S3131, D16S753, D16S752, D19S591, D19S426, D19S891, and D19S214 (in the order from p arm to q arm). STR, short tandem repeat.

To evaluate whether these triploid digynic miscarriages resulted from the failure of Meiosis I (MI) or Meiosis II (MII), we investigated the segregation of alleles at 14 STR markers located in the vicinity of the centromeres. We identified six markers, from six different chromosomes, at which the mother is informative (heterozygous), and that were located at less than 7.4 Mb from the centromeres (Figure 2.1; Table S2.2). Analysis of these markers showed the transmission of only one maternal allele to each of POC7 and POC8 with two exceptions, one in POC8 and one in POC7 at markers D10S1208 and D21S1436 located at 4.8 Mb and 7.4 Mb from the centromeres, respectively. These data suggested that the maternal triploidies are caused by the failure of the separation of sister chromatids at MII.

Altered number and distribution of crossovers have been associated with aneuploid miscarriages. To determine whether this is the case in the two triploid digynic conceptions of our patient, we performed SNP microarray analysis on DNA from POC7 and POC8, along with parental DNA. The analysis of the SNP microarray data on parental DNA confirmed their normal karyotypes without any detectable abnormality. The analysis of the two POCs confirmed their triploidies by the presence of four-allele peak tracks on all autosomes (AAA, AAB, ABB, and BBB; Figure S2.1). To map the positions of the maternal crossing overs, we filtered for positions where the father was homozygous, and looked at sites where the patient is informative and has two

different alleles (Figure S2.2). Using this analysis, we found that a total of 60 crossing overs occurred in POC7 and 62 in POC8 (Figure 2.1). These numbers when divided by two to correct for the two sets of maternal chromosomes in triploid digynic conceptions, give a total of 30 and 31 crossing overs, which is at the lower limit of the average number of crossing overs in female meiosis per haploid set of chromosomes, estimated to about 41.6 ± 11.3 (Lynn et al., 2004; Ottolini et al., 2015; Wirtenberger et al., 2005). Our SNP microarray analysis confirmed the failure of MII but revealed additional meiotic abnormalities in POC8. Judging by the allele type at the centromeric regions, this POC has received the two maternal homologous of chromosomes 16 and 19. On chromosome 19, we were able to observe one crossover event where maternal heterozygosity was reduced to homozygosity in POC8. For chromosome 16, we did not see a reduction to homozygosity, which implies either the absence of crossovers or the presence of the two reciprocal recombining chromatids which will appear as retained heterozygosity at all loci. These SNP microarray data were also confirmed using five and four informative STR markers from chromosomes 16 and 19.

Differentially methylated regions (DMRs) are associated with parent-of-origin specific transcription. In the female, these methylation marks are established during oocyte maturation, which takes place after the meiotic prophase I and before the completion of MII after fertilization. To investigate whether other meiotic abnormalities occurred in her two conceptions, POC7 and POC8, we assessed de novo DNA methylation at DMRs of imprinted regions using the Illumina Human Methylation-EPIC microarray. This analysis did not reveal statistically significant hypomethylation at maternally methylated DMRs (Figure S2.3) or genome-wide (within the limitation of the used methylation microarray) for the two POCs (Figure S2.4). Our data are in agreement with previous observations (Bourque et al., 2011) and showed a slightly

higher level of DNA methylation in the two triploid digynic conceptions as compared to diploid biparental ones. This is due to the presence of DNA methylation on the two copies of the maternal genome, which represent 2/3 (67%) of the total copy numbers, whereas in a diploid biparental conception, maternal DNA methylation represents 1/2 (50%) of the total copy numbers (Bourque et al., 2011). This analysis expands the number of available methylation data on triploid digynic POCs that could be useful to interpret placental abnormalities in future studies.

Whole exome sequencing analysis

The maternal origin of two triploid miscarriages suggested a possible germline genetic defect in the patient at the origin of her RM. Because of the absence of any form of reproductive failure in the patient's parents and grand-parents, a recessive maternal defect was prioritized. We therefore performed WES on the patient's DNA. Identified variants were filtered for the following criteria: (1) homozygous, possible homozygous, or multiple heterozygous variants with minor allele frequencies less than 0.01 in gnomAD (v2.1.1 https://gnomad.broadinstitute.org/; Karczewski et al., 2020); (2) variants that are absent or very rare in 4400 in-house WES controls; (3) variants that correspond to stop gain, stop loss, invariant splice sites, frameshift insertion or deletion, or conserved missense variants with Combined Annotation Dependent Depletion (CADD) scores ≥10 (https://cadd.gs.washington.edu/; Rentzsch et al., 2021); and (4) variants in genes that are not highly mutated in in-house WES. Variants fulfilling these criteria were further investigated for their potential role in female reproduction (Figure S2.5). Two candidate genes met the above criteria and were validated by Sanger sequencing. Both variants were in the same run of homozygosity of 10 Mb on chromosome 22: a missense variant, c.554G>A, p.Arg185Gln in exon 5 of *EIF4ENIF1* and a stop gain variant, c.505C>T, p.Gln169* in exon 9 of *HORMAD2*

(Figure 2.2). Furthermore, WES analysis of the male partner DNA and filtering his variants under the recessive mode did not reveal any variant that fulfill the above described criteria and that has a possible role in male reproduction (Figure S2.6).

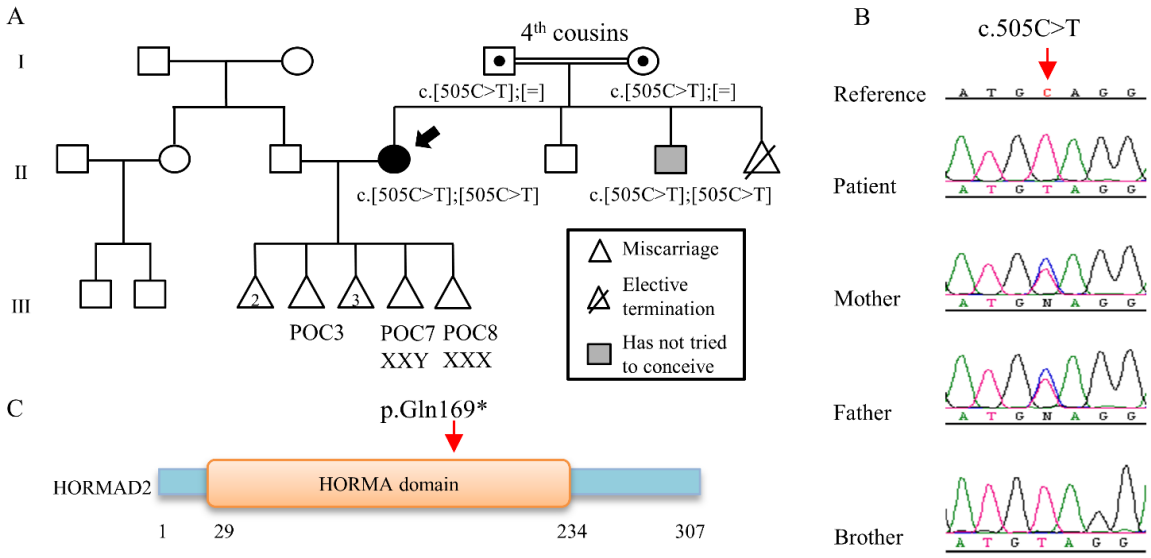

Figure 2.2 A stop codon variant in *HORMAD2* in a patient with eight recurrent miscarriages

(a) Pedigree and reproductive history of the patient. (b) Sanger sequencing confirmation of the stop codon variant in the patient, her parents and brother. *HORMAD2* variant is provided in Reference Sequence NM_152510.4. (c) Location of the identified mutation on HORMAD2 protein structure adapted from UniProt annotation (https://www.uniprot.org/uniprotkb/Q8N7B1/entry#family_and_domains; The UniProt Consortium, 2023).

Analysis of the DNA from her two parents and one of her brothers showed that the parents are heterozygous carrier of the two variants in *HORMAD2* and *EIF4ENIF1* while the brother has the same genotype as the patient (Figure 2.2). However, the fertility status of the brother is unknown since he has not tried to conceive. The *EIF4ENIF1* variant has an allele frequency of 0.0000159 in gnomAD Exomes (Karczewski et al., 2020) with 4 European non-Finnish males carrying this variant in heterozygous state and no reported homozygotes. This variant was predicted to be a variant of unknown significance (VUS) by the ACMG guidelines (Richards et al., 2015). The variant in *HORMAD2* has an allele frequency in gnomAD Exomes (Karczewski et al., 2020) of 0.000556 with a total of 137 individuals carrying this variant in a heterozygous state and only two in a homozygous state (both South Asian males with no data about their reproduction). This variant frequency appears to be highest in South and East Asian populations and is predicted by the ACMG guidelines to be likely pathogenic (Richards et al., 2015). We next screened the exome data of 177 patients with RM but did not identify any patient with recessive variants in any of the two genes.

2.5 Discussion

In this study, we report the genotypic analysis of two POCs from a female patient with eight RM and no live birth. Using simplex and multiplex STR genotyping, we demonstrated that both POCs were triploid digynic and have resulted from the failure of maternal MII. SNP microarray analysis confirmed the failure of maternal MII and revealed the presence of the two sister chromatids for all the chromosomes except for chromosomes 16 and 19, for which the two maternal homologs segregated in one of the two analyzed POCs. Analysis of DNA methylation

confirmed that both POCs were triploid digynic, but did not reveal any additional DNA methylation abnormalities at imprinted DMRs.

WES revealed two homozygous variants, a missense VUS, p.Arg185Gln in *EIF4ENIF1*, and a likely pathogenic nonsense p.Gln169* in *HORMAD2* segregating on the same haplotype from her two parents. We failed to identify a second patient with mutations in any of the two genes in a cohort of 177 patients with RM. This is not unexpected because RM is a highly heterogeneous entity, and from our experience, most are not caused by a recessive maternal genetic defect. *EIF4ENIF1* codes for the eukaryotic translation initiation factor 4E nuclear import factor. In Drosophila, EIF4ENIF1 interacts with another protein to modulate ovarian development (Zappavigna et al., 2004). In mouse oocytes, knocking-down *Eif4enif1* leads to failure of the oocyte nuclear envelop breakdown and oocyte development arrest (Pfender et al., 2015). To date, five different single-heterozygous variants in *EIF4ENIF1* segregating in an autosomal dominant manner have been found in patients with premature ovarian insufficiency (POI; França et al., 2020; Kasippillai et al., 2013; Shang et al., 2022; Zhao et al., 2019). Four of these variants are missenses predicted by the ACMG (Richards et al., 2015) to be benign/likely benign, and only one is protein-truncating predicted to be VUS and segregating with the phenotype in a large family. However, no functional studies have yet demonstrated that these monoallelic variants cause POI. The fact that neither our patient nor her mother have had any feature of POI (amenorrhea, small ovarian volume, low AMH, low antral follicle count) and that the patient's mother had her menopause at the age of 55 years make it unlikely for the *EIF4ENIF1* variant in our patient to be the cause of her RM.

HORMAD2 mutations have not been associated with reproductive failure in humans. However, there has been extensive research regarding HORMAD2 functions in knockout mouse models.

HORMAD2 is part of the synaptonemal complex (SC), which is a proteinaceous structure present only in meiotic cells (Xie et al., 2022). The SC consists of two axial elements that form along the longitudinal axes of sister chromatid pairs and a central element. Sister chromatids are bound to the axial elements and to each other via cohesin complex. During prophase I, HORMAD2 localizes mainly to unsynapsed regions of the axial elements and has a supporting role in completing synapsis (Kogo et al., 2012). Additionally, HORMAD2 plays an essential role in efficient ATR recruitment to unsynapsed chromatin, H2AX phosphorylation, and meiotic silencing of unsynapsed chromatin (Kogo et al., 2012; Wojtasz et al., 2012). Hormad2$^{-/-}$ male mice are infertile because the lack of HORMAD2 in the presence of normal asynapsis between the largely nonhomologous X and Y chromosomes leads to spermatocyte apoptosis due to impaired meiotic sex chromosome inactivation. In females, asynapsis does not naturally occur on sex chromosomes, and consequently, HORMAD2 deficiency is tolerated, and the females are fertile and have normal litter sizes. The only abnormalities observed in their oocytes are a slight increase in the number of incomplete synapsis in fetal ovaries and a slight decrease in the frequency of chiasmata formation in *in vitro* matured metaphase I oocytes in adult mice, as compared to wildtype. Incomplete synapsis is well-known to be associated with a lower than normal number of crossing overs, which is in agreement with the borderline low number of crossing overs observed in the two triploid POCs of our patient. Since HORMAD2 in mice is thought to be involved in the elimination of oocytes with asynapsis via a checkpoint mechanism, it is possible that its recessive mutation in our patient prevented her oocytes with synaptic errors from being eliminated. These oocytes progressed to MII, were fertilized, but failed to extrude the second polar body. Alternatively, the abnormal segregation of the two homologous chromosomes 16 and 19 in POC8 may have been caused by the lack of normal HORMAD2 from

the axial elements of the SC, which may have impaired sister chromatid attachment by cohesin complex and led to their precocious separation. This followed by the random segregation of their four chromatids at MI may have led to the presence of the two homologous chromosomes 16 and 19 in POC8 (Handyside et al., 2012; Ottolini et al., 2015). This suggestion is in line with the increase of univalents in *Hormad2*$^{-/-}$ oocytes (Wojtasz et al., 2012), which is well-documented to promote the precocious separation of sister chromatids in MI (Capalbo et al., 2017). Also, the presence of two triploid conceptions in our patient is in agreement with the high prevalence of hyperploidy observed in embryos derived from null-female mice for HORMAD1, which co-localizes and interacts with HORMAD2 (Kim et al., 2014; Wojtasz et al., 2009, 2012).

Human and murine HORMAD2 proteins are highly conserved with 76% of identity and 87% of similarity. While *Hormad2*-deficient female mice are fertile and have normal litter sizes, our patient did not achieve any live birth, which suggests potential differences in how HORMAD2 functions in mice and humans. It is also possible that *Hormad2*-deficiency might have a more severe impact on female fertility in other mouse strains than in the strain studied by Wojtasz et al. (2012) and Kogo et al. (2012).

Although the male partner had mild abnormalities in the semen analysis, his karyotype by culture-based cytogenetic did not reveal any chromosomal abnormality, which was also confirmed by SNP microarray analysis. WES analysis on his DNA did not reveal any plausible causative candidate variant. The fact that both analyzed POCs are digynic triploid is in support of a maternal genetic defect underlying the eight consecutive miscarriages.

In summary, while we cannot exclude a possible contribution of the *EIF4ENIF1* variant to the phenotype of the patient, the presence of MI and MII abnormalities in two of her conceptions are in favor of the causative role of *HORMAD2* variant, which remains to be confirmed in more

patients in future studies. Our study highlights the genetic complexity of RM and the importance of SNP microarray in determining the meiotic origin of triploid conceptions and guiding the search for their causative genes.

AUTHOR CONTRIBUTIONS

Manqi Liang and Rima Slim: Planning and conducting the project, data analysis, and drafting the manuscript. Beena Suresh and Sujatha Jagadeesh: Referring the patient, gathering clinical information and materials from the conceptions. Eric Bareke and Jacek Majewski: Processing raw data of exome sequencing and conducting variant calls. Sanaa Choufani and Rosanna Weksberg: performing the methylation microarray and guiding its analysis. All authors read and commented on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

This study involves human participants and was approved by McGill University Faculty of Medicine and Health Sciences (A01-M07-03A) in 2003 and renewed yearly since. Participants gave written informed consent to participate in the study.

Open Research

DATA AVAILABILITY STATEMENT

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

Supporting Information

Data S1:

2.6 Supplemental material

Supplementary Table S2.1 Primers designed to validate candidate variants via PCR and Sanger Sequencing. NCBI Reference Sequence for *HORMAD2* is NM_152510.4, and for *EIF4ENIF1* is NM_019843.4.

Supplementary Table S2.2 Table summarizing data on the 16 Multiplex and six informative Simplex STR markers used for genotyping the patient, her partner, POC7 and POC8. Alleles received from father (partner) are in blue. Alleles received from mother (patient) are in red. Underlined alleles in red indicate loci where two copies of the same maternal allele were inherited.

Supplementary Figure S2.1 Beta allele frequency obtained from SNP microarray of the patient, her partner, POC7 and POC8 on all chromosomes.

Supplementary Figure S2.2 Illustration of possible meiotic recombination outcome at a single locus of the SNP microarray where the patient has two different alleles and the partner has one single allele.

Supplementary Figure S2.3 Methylation values in chorionic villi from two control samples from elective termination of pregnancies (diploid biparental genomes) and from POC7 and POC8 (triploid digynic genomes) at maternally methylated differentially methylated regions (DMRs).

Supplementary Figure S2.4 Histograms of Methylation values for POC7, POC8 and three diploid biparental control samples from first trimester elective termination of normal pregnancies.

Supplementary Figure S2.5 Schematic of the analytical workflow of variant filtering for the female patient with the criteria on the left. Variant pathogenicity prediction was performed according to ACMG guidelines (Richards et al., 2015) using VarSome (https://varsome.com/) (Kopanos et al., 2019). Abbreviations: LP-likely pathogenic, VUS-variant of unknown significance, B-benign, LB-likely benign. The 11 genes that remained after the initial filtering steps are listed below. The two genes with roles in female reproduction are highlighted in green and selected for validation and segregation by Sanger.

Supplementary Figure S2.6 Schematic of the analytical workflow of variant filtering for the male partner with the criteria on the left. Variant pathogenicity prediction was performed according to ACMG guidelines (Richards et al., 2015) using VarSome (https://varsome.com/) (Kopanos et al., 2019). Abbreviations: LP-likely pathogenic, VUS-variant of unknown significance, B-benign, LB-likely benign. The nine genes that remained after the initial filtering steps are listed below. Among these genes, none has function in male reproduction.

Table S2.1

Primers	Sequences
HORMAD2-EX9Forward	agtaccatcgctcccctaattt
HORMAD2-EX9Reverse	agctttgccttacATGGTCATT
EIF4ENIF-EX5Forward	TAGATGTTGTTCTCAGCCCTCA
EIF4ENIF-EX5Reverse	ttcctccctgactggtttaaga

Table S2.2

STR	Chr	Patient	POC7	POC8	Partner
Powerplex makers					
Penta E	15	387/434	387/413	387/434/455	413/455
D8S1179	8	234/238	230/234/238	214/234/238	214/230
D13S317	13	180/200	180/196/200	180/196/200	192/196
Amelogenin	X/Y	104(XX)	104/110(XXY)	104/104(XXX)	104/110(XY)
vWA	12	146	146/154	146/154	146/154
TPOX	2	269/281	269/281	269/281/281	281
FGA	4	350/358	350/350/358	346/350/358	346/350
D3S1358	3	124	124/124	124/133	124/133
TH01	11	174	170/174	170/174	166/170
D21S11	21	219/237	219/219/237	215/219	215/219
D18S51	18	302/306	302/314	302/306/314	314
D5S818	5	131	127/131	131/135	127/135
D7S820	7	228/236	228/228/236	228/236	228
D16S539	16	283	283/287	283/295	287/295
CSF1PO	5	341/345	333/341	341/341/345	333/341
Penta D	21	403/413	403/413	403/413/413	413/417
Simplex markers					
D1S534	1	203/207	207/207	199/203	199/207
D3S2462	3	233/241	233/237	233/237	237/239
D11S1983	11	237/241	237/241	222/237	222/241
D20S484	20	198/202	182/202	182/198	182/194
D10S1208	10	183/198	177/198	183/189/198	177/189
D21S1436	21	170/182	166/170/182	166/182	166

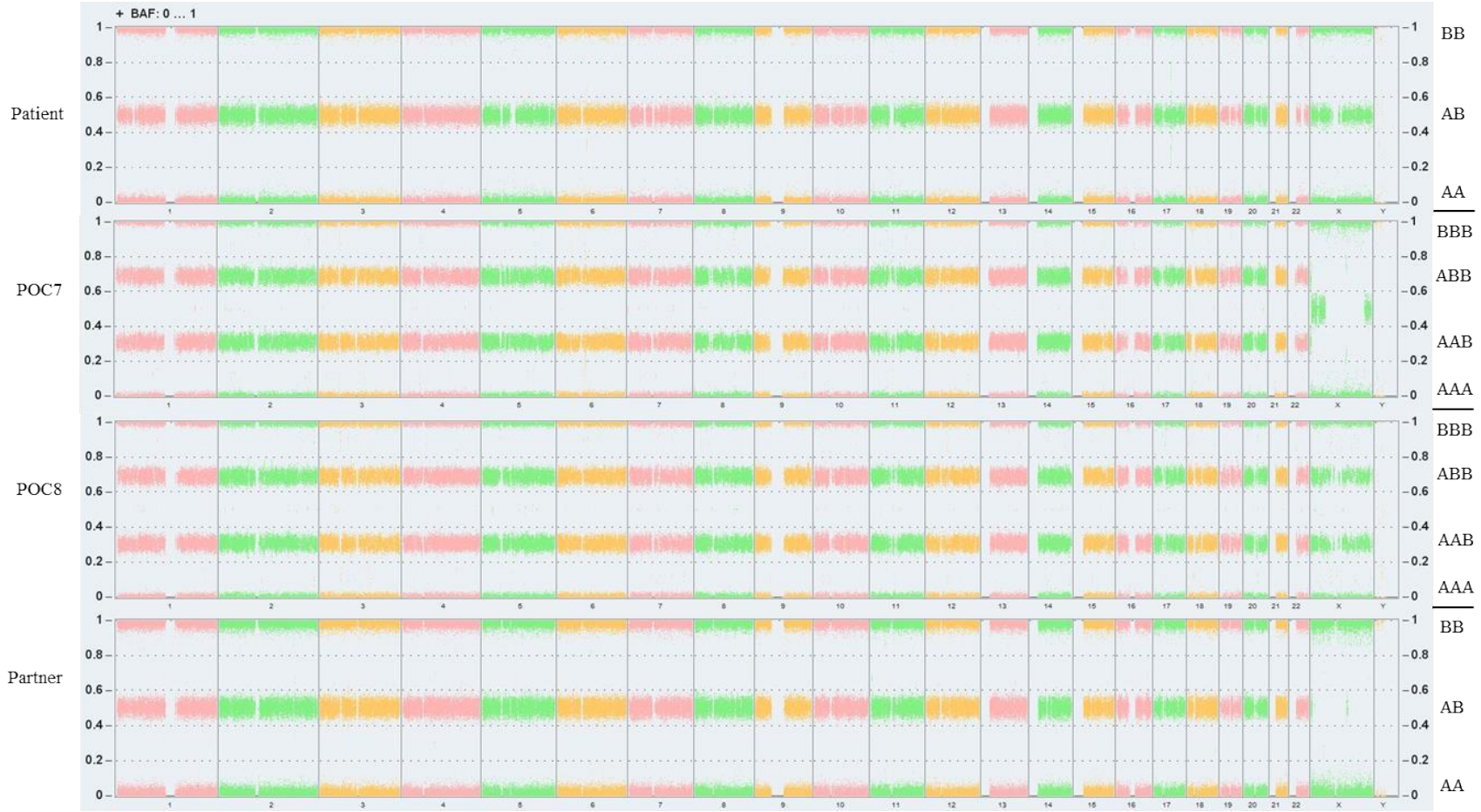

+ BAF: 0 ... 1
Patient
POC7
POC8
Partner
BB
AB
AA
BBB
ABB
AAB
AAA
BBB
ABB
AAB
AAA
BB
AB
AA

Figure S2.1

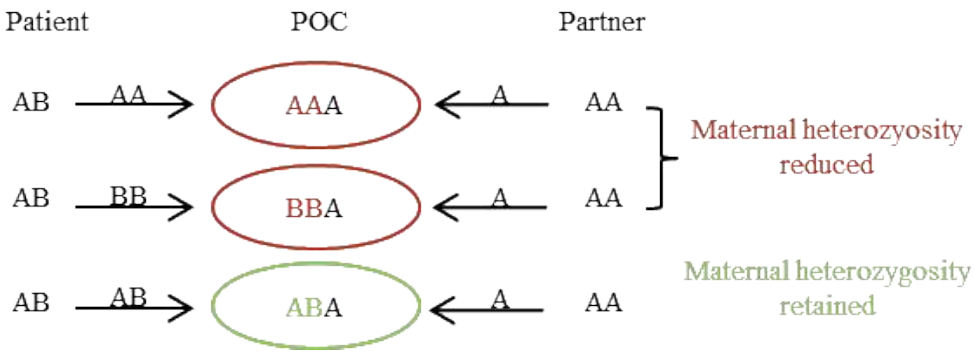

Patient
POC
Partner
AB
AA
AAA
A
AA
AB
BB
BBA
A
AA
Maternal heterozyosity reduced
AB
AB
ABA
A
AA
Maternal heterozygosity retained

Figure S2.2

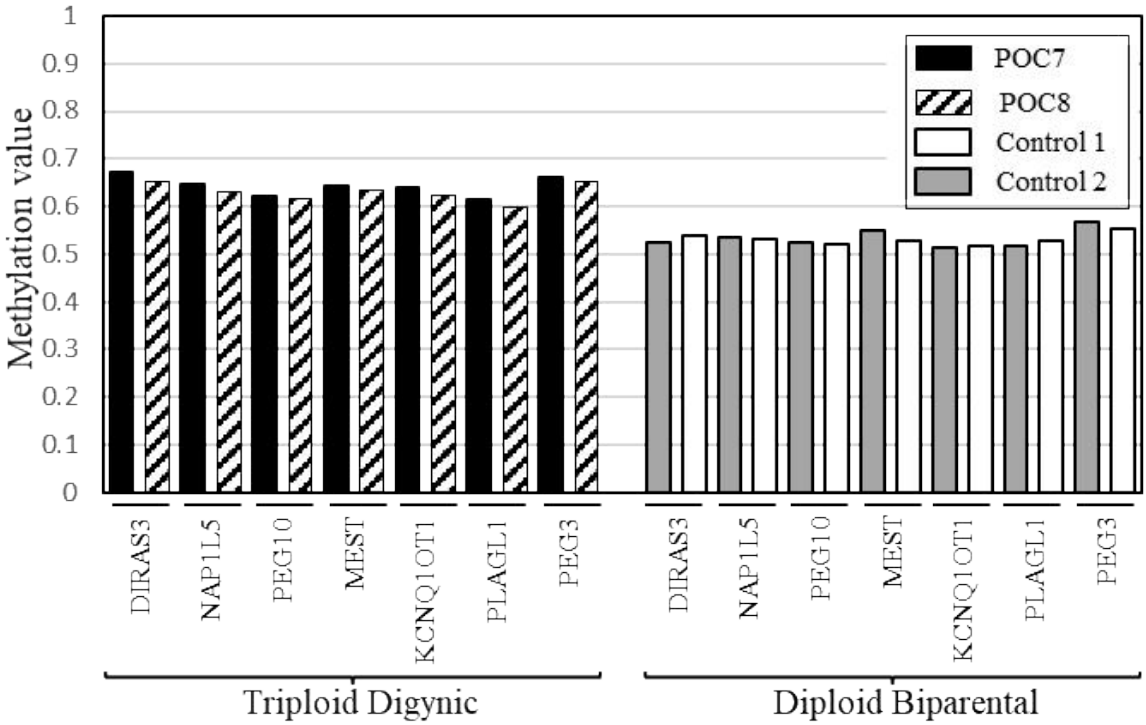

Methylation value
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
POC7
POC8
Control 1
Control 2
DIRAS3
NAP1L5
PEG10
MEST
KCNQ1OT1
PLAGL1
PEG3
DIRAS3
NAP1L5
PEG10
MEST
KCNQ1OT1
PLAGL1
PEG3
Triploid Digynic
Diploid Biparental

Figure S2.3

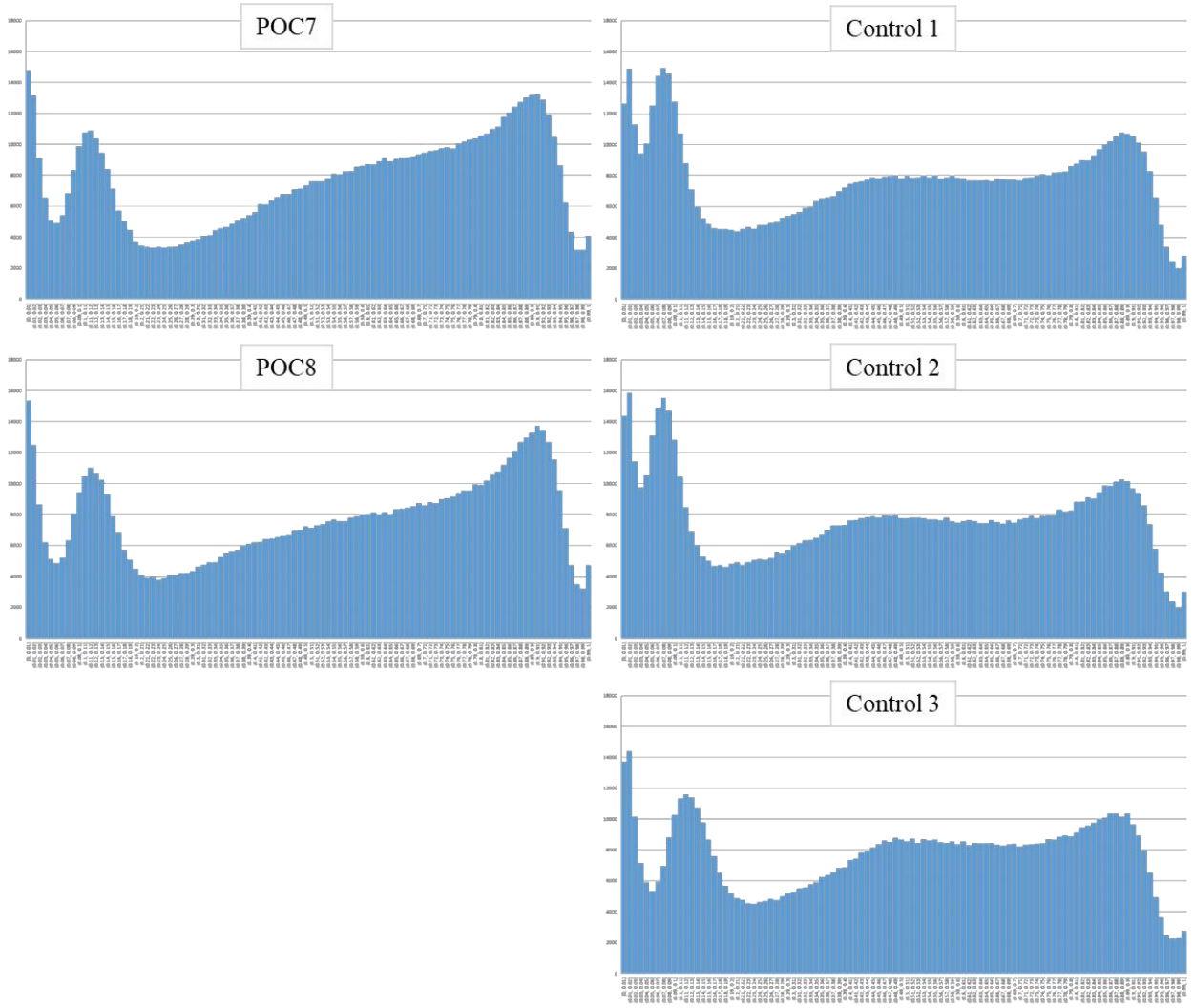
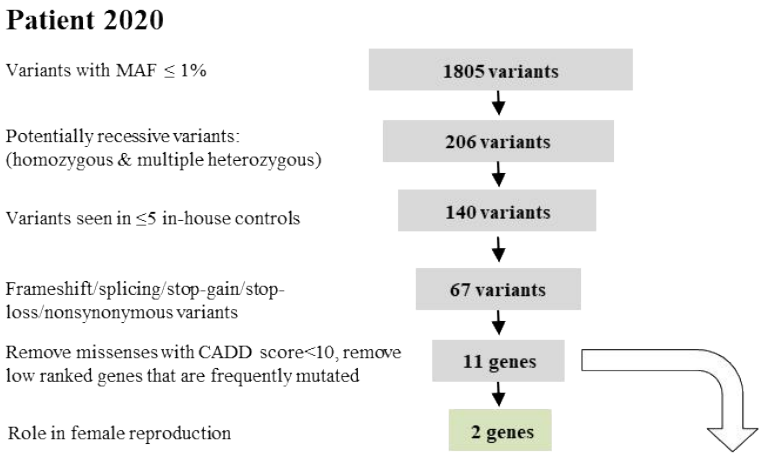
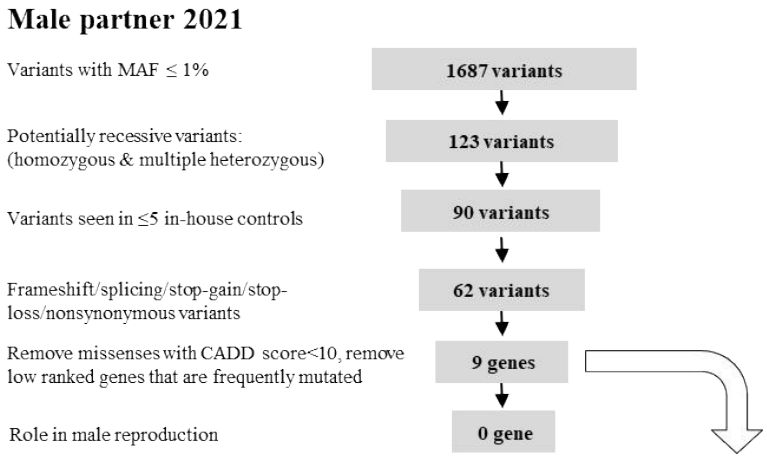


Figure S2.4



Position	Variation	Zygosity	Protein Change	Gene	CADD score	Gene Description	ACMG prediction
chr1:31230540	nonsynonymous SNV	hom	p.R18H	LAPTM5	32.0	lysosomal associated multispanning membrane protein 5	B
chr1:43919306	frameshift insertion	hom	p.R53fs	HYI	0	hydroxypyruvate isomerase homolog (E. coli)	VUS
chr2:236877166	nonsynonymous SNV	multiple het	p.K462T	AGAP1	25.2	centaurin, gamma 2	LB
chr2:236877170	frameshift insertion	multiple het	p.L463fs	AGAP1	0	centaurin, gamma 2	LP
chr3:120067667	nonsynonymous SNV	multiple het	p.L142M	LRRC58	19.8	leucine rich repeat containing 58	B
chr3:120067961	nonsynonymous SNV	multiple het	p.E44K	LRRC58	23.4	leucine rich repeat containing 58	LB
chr6:32489754	frameshift insertion	possibly hom	p.R100fs	HLA-DRB5	0	major histocompatibility complex, class II, DR beta 5	B
chr6:170036584	nonsynonymous SNV	hom	p.S438Y	WDR27	15.3	WD repeat domain 27	B
chr12:132625896	nonsynonymous SNV	multiple het	p.A392T	DDX51	21.9	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	LB
chr12:132627396	nonsynonymous SNV	multiple het	p.A183P	DDX51	23.7	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	LB
chr19:44891401	nonsynonymous SNV	hom	p.R181G	ZNF285	20.8	zinc finger protein 285A	LB
chr22:30517715	stopgain	hom	p.Q169X	**HORMAD2**	39.0	HORMA domain containing 2	LP
chr22:31859698	nonsynonymous SNV	hom	p.R185Q	**EIF4ENIF1**	34.0	eukaryotic translation initiation factor 4E nuclear import factor 1	VUS
chr22:32111832	nonsynonymous SNV	hom	p.N665D	PRR14L	10.1	Proline Rich 14 Like	LB

Figure S2.5



Position	Variation	Zygosity	Protein Change	Gene	CADD score	Gene Description	ACMG prediction
chr1:54605318	frameshift insertion	hom	p.M409fs	CDCP2	0	CUB domain containing protein 2	VUS
chr8:145163244	nonsynonymous SNV	multiple het	p.A92V	WDR97	18.56	WD Repeat Domain 97	LB
chr8:145163300	nonsynonymous SNV	multiple het	p.R111C	WDR97	12.9	WD Repeat Domain 97	VUS
chr15:30010828	nonsynonymous SNV	multiple het	p.P1093L	TJP1	20.1	tight junction protein 1 (zona occludens 1)	B
chr15:30025427	nonsynonymous SNV	multiple het	p.S536T	TJP1	27.5	tight junction protein 1 (zona occludens 1)	B
chr15:91169159	nonsynonymous SNV	multiple het	p.S301C	CRTC3	24.6	CREB regulated transcription coactivator 3	LB
chr15:91181742	nonsynonymous SNV	multiple het	p.P444L	CRTC3	25	CREB regulated transcription coactivator 3	LB
chr19:45682654	nonsynonymous SNV	multiple het	p.S34A	BLOC1S3	11.13	biogenesis of lysosome-related organelles complex-1, subunit 3	LB
chr19:45682655	stopgain	multiple het	p.S34X	BLOC1S3	36	biogenesis of lysosome-related organelles complex-1, subunit 3	LP
chr22:43579002	nonsynonymous SNV	multiple het	p.A111T	TTLL12	23.6	tubulin tyrosine ligase-like family, member 12	LB
chr22:43583014	nonsynonymous SNV	multiple het	p.P21L	TTLL12	12.2	tubulin tyrosine ligase-like family, member 12	LB
chrX:84349207	stopgain	hom	p.Y601X	SATL1	43	spermidine/spermine N1-acetyl transferase-like 1	B
chrX:101970092	nonsynonymous SNV	hom	p.K99Q	GPRASP2	19.23	G protein-coupled receptor associated sorting protein 2	B
chrX:152935958	nonsynonymous SNV	hom	p.R412H	PNCK	21.2	pregnancy upregulated non-ubiquitously expressed CaM kinase	LB

Figure S2.6

2.7 References

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3 GENERAL DISCUSSION

As mentioned in Section 1.5, errors in the maternal Meiosis or oocyte development may cause adverse defect in the embryo genome. Importantly, synaptic and recombination failures have been extensively implicated in generating aneuploid embryos or causing infertility in both females and males (Adams & Davies, 2023; Hollingsworth, 2020; Xie et al., 2022; Yuan et al., 2002; F. Zhang et al., 2022). The SC is a highly conserved meiosis-specific protein structure that plays crucial role in physically linking homologous chromosomes and facilitating CO formation during meiotic Prophase I. The structural components that make up the tripartite SC includes the two lateral elements (LEs) and central elements (CEs), which are connected by the transverse filaments (TFs) constituted by SYCP1 (Adams & Davies, 2023; Llano & Pendás, 2023; Xie et al., 2022). The two parallel LEs are referred as axial elements (AEs), which form during leptotene prior to the loading of CE and TFs. The AEs are constituted by SYCP2 and SYCP3, which interact with each other to form a scaffold along the chromosome axis for chromatin loop extrusion. SYCE1/2/3, SIX6OS1, and TEX12 make up the CE and provide stable connection between the TFs. The cohesin complex is a ring-shaped structure that is essential for keeping the sister chromatids together in mitosis and meiosis (Xie et al., 2022; F. Yang et al., 2006). Core subunits of the cohesin complex includes SMC1α/β, SMC3, SA1/SA2. STAG3, REC8, RAD21, and RAD21L1. Among these proteins, SMC1β, STAG3, RAD21L1 and REC8 are the meiosis specific subunits. The HORMA-domain proteins HORMAD1/2 preferentially localize to unsynapsed chromosome axes to promote DSB repair and regulate surveillance of synapsis (Kogo, Tsutsumi, Inagaki, et al., 2012; Wojtasz et al., 2012). TRIP13 removes HORMAD1/2 from chromosomes after completion of synapsis (Wojtasz et al., 2009). The role of these proteins in mouse reproduction are summarized in Table 3.1.

Table 3.1 Synaptonemal complex (SC) associated proteins and their roles in mouse reproductive functions

Protein name	Meiotic role	Reproductive phenotype of knockout (KO)/mutated mice	References
Axial elements (AEs) of SC			
SYCP2	Forms heterodimers/oligomers with SYCP3. Required for the incorporation of SYCP3 into AEs, synaptonemal complex assembly, and synapsis.	Homozygous deletion of the coiled coil domain leads to infertility in males, while females have reduced litter sizes.	(F. Yang et al., 2006)
SYCP3	Forms complex with SYCP2. Required for the formation of AEs. Involved in DNA compaction by stabilizing chromatin loops.	KO males are infertile. KO females can give live births, but have a higher rate of intrauterine death due to aneuploid oocytes, which increases with advanced maternal age. Around half of the oocytes at Metaphase I from mutant females contain univalents.	(Yuan et al., 2000, 2002)
Transverse filaments (TFs) of SC			
SYCP1	Provides the structural basis for meiotic chromosome synapsis. Required for the formation of the XY body in pachytene spermatocytes.	KO males and females are both infertile.	(F. A. T. de Vries et al., 2005)
Central elements (CEs) of SC			
SYCE1	Required for stabilization of SYCP1 association, regulation of TFs stacking formation, and synaptonemal complex extension.	KO males and females are both infertile.	(Bolcun-Filas et al., 2009)
SYCE2	Required for elongation of the synaptonemal complex and XY body formation.	KO males and females are both infertile.	(Bolcun-Filas et al., 2007)
SYCE3	Required for loading of other central element proteins and progression of recombination.	KO males and females are both infertile.	(Schramm et al., 2011)
SIX6OS1	Interacts with SYCE1. Required for processing of intermediate recombination nodules before crossover formation and XY body formation.	KO males and females are both infertile.	(Gómez-H et al., 2016)
TEX12	Interacts with SYCE2. Required for the elongation of the synaptonemal complex.	KO males and females are both infertile.	(Hamer et al., 2008)
Chromosome axis components			
HORMAD1	Accumulates on unsynapsed chromosomes. Required for the recruitment of BRCA1, ATR and γH2AX and the checkpoint mechanism to eliminate asynaptic oocytes. May recruit HORMAD2 to the axis.	KO male and female mice are both infertile. Aneuploidy in embryos from mutant oocytes causes embryonic development arrest at blastocyst stage.	(Kogo, Tsutsumi, Ohye, et al., 2012; Shin et al., 2010)
HORMAD2	Localizes on unsynapsed chromosome axis and monitor homolog synapsis. Required for the accumulation of ATR along unsynapsed axis and to prevent DSB repair via intersister recombination.	KO males are infertile. KO females are fertile, but the frequency of univalent-containing metaphase I oocytes in fetal ovaries is 4.13%, slightly higher than 0.52% in wildtype.	(Kogo, Tsutsumi, Inagaki, et al., 2012; Rinaldi et al., 2017; Wojtasz et al., 2009, 2012)
Meiosis specific cohesin complex			
SMC1β	Required for normal chromosome axis length (by restricting chromosome compaction that is exerted by AE), regulating loop length, and telomere integrity. Promotes closed chromatin near telomeres in spermatocytes.	KO males and females are both infertile.	(U. Biswas et al., 2023; Revenkova et al., 2004; Takabayashi et al., 2009)
STAG3	Forms complex with SMC1α/β. Required for chromosome axis formation and sister chromatid cohesion and the stability of all meiosis-specific cohesin complexes.	KO males and females are both infertile.	(Hopkins et al., 2014; Winters et al., 2014)
RAD21L1	Promotes recombination between homologous chromosomes. Required for normal chromosome axis length (by restricting chromosome compaction that is exerted by AE). Proposed to mediate chromatin loading and the mode of action of HORMAD1.	KO males are infertile. KO females can give live births, but have premature onset of subfertility around six months, and become infertile around 10 months.	(Fujiwara et al., 2020; Herrán et al., 2011; J. Lee & Hirano, 2011)
REC8	Required for sister chromatid cohesion and normal chromosome axis length (by restricting chromosome compaction that is exerted by AE), and prevents illegitimate synaptonemal complex formation. Proposed to mediate chromatin loading and the mode of action of HORMAD1.	KO males and females are both infertile, with *in utero* and postnatal growth retardation and high mortality rate.	(Agostinho et al., 2016; Fujiwara et al., 2020; Sakuno et al., 2022; H. Xu et al., 2005)

Sexual dimorphism of fertility in KO mice with deficiencies in these proteins has been observed, where the phenotypes in males are sometimes more severe than in females, consistent with the general consensus about the leakiness of the SAC in female meiosis (Lane & Kauppi, 2019). Although there is evidence for leakiness in male meiotic SAC, synaptic failures in spermatogenesis more frequently lead to spermatocyte apoptosis and consequently male infertility. Whereas in females, not all defective oocytes are necessarily eliminated, some might experience delay in meiotic progression but can still get fertilized, and lead to aneuploid embryos. For instance, deficiencies in SYCP2/3, HORMAD2, and RAD21L1 lead to infertility in males while the females can still produce live births, but have reduced litter sizes or other abnormalities (Herrán et al., 2011; Kogo, Tsutsumi, Inagaki, et al., 2012; Wojtasz et al., 2012; F. Yang et al., 2006; Yuan et al., 2000, 2002). Another possible explanation of the phenotypic sexual dimorphism is that the SC structure in females is narrower, and have shorter chromatin loops and longer chromosome axes, which has been proposed to lead to the higher rate of recombination in oocytes in comparison to spermatocytes (Adams & Davies, 2023; Tease & Hultén, 2004).

Over the past two decades with the advancement of next-generation sequencing technologies, an increasing number of defects in meiosis related genes have been linked to human reproduction failure. Among the key members of the SC and chromosome axis related components, mutations in *SYCP1/2/3*, *SYCE1*, *SIX6OS1*, *TEX12*, *HORMAD1*, *STAG3*, *RAD21L1* and *REC8* have been associated with human infertility and/or RM (Table 3.2 & Table 3.3). I summarized and evaluated all reported variants in these genes using the ACMG guidelines through Varsome (https://varsome.com/) (Kopanos et al., 2019) and Franklin (https://franklin.genoox.com), and gave the evidence level for the causative role of each gene. My analysis showed that autosomal

recessive (AR) pathogenic variants in *SYCE1*, *SIX6OS1*, *STAG3* and *REC8* cause infertility in both women and men with strong evidence. Evaluation of the consequences of each variant on mouse reproduction will provide a better understanding of how they lead to the reproductive phenotypes observed in humans. A protein-truncating AR variant in these SC-associated genes in patients with RM is solely reported in our study in Chapter 2. The scarcity of reported recessive pathogenic variants in meiotic genes in patients with RM is partially due to the higher heterogeneity of RM as compared to infertility, which highlights the difficulties in the identification of their causative genes. In the future, I expect to see additional recessive pathogenic variants in *HORMAD2* to be found in women with RM due to embryonic aneuploidy and perhaps with some conceptions showing MI and/or MII errors. I also believe that some patients with recessive pathogenic variants in *HORMAD2* may achieve normal pregnancies and live births in some of their conceptions. Additionally, I expect that recessive pathogenic *HORMAD2* variants in men will cause infertility due to azoospermia or milder sperm abnormalities.

Table 3.2 Genes associated with structural components of the synaptonemal complex and their roles in human reproductive functions

Gene name	Variant zygosity	Protein change	Phenotype		Ethnicity	Patients analyzed	References	My evaluation		
			Women (N. & relationship)	Men (N. & relationship)				ACMG prediction	Evidence	Comments
SYCP1	c.2892delA hom	K967Nfs*2	/	oligozoospermia (3 brothers)	Iranian	/	(Nabi et al., 2022)	LB/VUS	2	AR impairs male fertility
SYCP2	c.2022_2025del het	K674Nfs*8	/	cryptozoospermia (1)	/	627 men with diverse infertility phenotypes	(Schilit et al., 2020)	P	1	AD may confer susceptibility, AR is more likely causative for male infertility
	c.2793_2797del het maternally inherited	K932Sfs*3	/	cryptozoospermia (1)	/			P		
	c.3067_3071del het	K1023Lfs*2	/	azoospermia (1)	/			P		
	c.2689_2690insT hom	A897Vfs*5	/	NOA (1)	Chinese	/	(J. Xu et al., 2023)	LP	2	
SYCP3	c.643delA het	I215Lfs*2	/	azoospermia (2 unrelated)	Hispanic/Arab	19 azoospermic men	(Miyamoto et al., 2003)	VUS	1	AD may confer susceptibility, AR is more likely causative for both sexes
	c.553-16_19del het	Intronic variant	3 MC, no live birth (1)	/	Japanese	26 women with ≥ 3 consecutive MC	(Bolor et al., 2009)	/	1	
	c.657T>C het	T219T	3 MC, no live birth (1)	/	Japanese			VUS		
	c.666A>G hom	Q222Q	infertility (2 unrelated)	/	Japanese	88 infertile women	(Nishiyama et al., 2011)	B	1	
	c.548T>C het	I183T	/	RM (1)	/	23 couples with ≥ 2 MC	(Stouffs et al., 2011)	VUS	1	
SYCE1	c.613C>T hom	Q205*	POI (2 sisters)	/	Arab	/	(L. de Vries et al., 2014)	VUS/P	3	AR causes infertility in both sexes
	c.197-2A>G hom	splicing	/	NOA (2 brothers)	Iranian Jewish	/	(Maor-Sagie et al., 2015)	LP	3	
	4 kb deletion hom	large deletion	POI (2 sisters)	/	Chinese	/	(Zhe et al., 2020)	/	3	
	c.375-2A>G hom	splicing	/	NOA (4 men in a family)	Iranian	/	(Pashaei et al., 2020)	VUS	3	
	c.1_1113del hom	Complete gene deletion	infertility (1 sister)	NOA (2 unrelated)	Venezuelan/ Chinese	17 men with NOA; 479 men with NOA	(An et al., 2021; Krausz et al., 2020)	/	3	
	c.689_690del; c.475G>A comp het	F230Sfs*21; E159K	POI (1)	/	Chinese	1030 women with sporadic POI	(Hou et al., 2022)	LP; VUS	3	
	c.271+2T>C hom	splicing	/	NOA (1)	Chinese	400 men with NOA		LP		
	c.689_690del hom	F230Sfs*21	/	NOA (1)	Chinese	/	(Feng et al., 2022)	LP	3	
SIX6OS1	c.204_205del hom	H68Qfs*2	POI (1 sister)	NOA (2 brothers)	Pakistanian	50 consanguineous Pakistani families with at least two infertile siblings and 60 infertile Chinese men with meiotic arrest	(Fan et al., 2021)	P	3	AR causes infertility for both sexes
	c.958G>T hom	E320*	/	NOA (1)	Chinese			P		
	c.1180-3C>G hom	splicing	/	NOA (1)	Chinese			VUS		
	c.508C>T hom	R170*	POI (1)	/	Chinese	1030 women with sporadic POI	(Hou et al., 2022)	LP	3	
	c.135_136del hom	K45Nfs*5	/	NOA (1)	Chinese	400 men with NOA		LP		
TEX12	c.196_200del hom	L66Vfs*10	/	NOA (2 brothers)	Vietnamese	/	(Bui et al., 2023)	VUS	2	AR causes NOA

Abbreviations: hom - homozygous; het - heterozygous; comp het - compound het; N. - number of individuals; MC - miscarriages; RM - recurrent miscarriages; POI - Primary ovarian insufficiency; NOA - non-obstructive azoospermia; VUS - variant of unknown significance; B - benign; LB - likely benign; P- pathogenic; LP - likely pathogenic; AD - autosomal dominant; AR - autosomal recessive. The American College of Medical Genetics and Genomics (ACMG) prediction was achieved by Varsome (https://varsome.com/) (Kopanos et al., 2019) and Franklin (https://franklin.genoox.com). Evidence level 1: weak evidence; 2: moderate evidence; 3: strong evidence.

Table 3.3 Genes associated with chromosome axis and cohesin complex during recombination and their roles in human reproductive functions

Gene name	Variant zygosity	Protein change	Phenotype		Ethnicity	Patients analyzed	References	My evaluation		
			Women (N. & relationship)	Men (N. & relationship)				ACMG prediction	Evidence	Comments
HORMAD1	c.1021C>T hom	Q341*	/	NOA (3 brothers)	Turkish	/	(Okutman et al., 2023)	P	3	AR causes NOA
HORMAD2	c.505C>T hom	Q169*	8 MC, no live birth (1)	/	Indian	177 women with RM	current study, 2024	LP/VUS	2	AR possibly causes RM
STAG3	c.968delC hom	Q188Rfs*8	POI (4 sisters and 1 aunt)	/	Middle Eastern	/	(Caburet et al., 2014)	P	3	AR causes infertility in both sexes
	c.1947_48dupCT hom	Y650Sfs*22	POI (2 sisters)	/	Lebanese	/	(Le Quesne Stabej et al., 2016)	P	3	
	c.677C>G hom	S226*	POI (2 sisters)	/	Asian	/	(Colombo et al., 2017)	LP	3	
	c.1573+5G>A hom	splicing	POI (2 sisters)	/	Chinese	/	(He et al., 2018)	LP	3	
	c.659T>G; c.3052delC comp het	L220R; R1018Dfs*14	POI (2 sisters)	/	Caucasian	/	(Heddar et al., 2019)	VUS; LP	3	
	c.877_885del; c.891_893dupTGA double hom	H293_E295del; I297_E298insD	POI (2 sisters)	/	Chinese	/	(Xiao et al., 2019)	VUS	3	
	c.291dupC; c.1950C>A comp het	N98Qfs*2; Y650*	POI (1)	/	Brazilian	48 sporadic POI cases	(França et al., 2020)	P	3	
	c.1759dupG; c.2394+1G>A comp het	A588Gfs*9; splicing	/	NOA (1)	/	33 men with NOA	(Riera-Escamilla et al., 2019)	P; LP	3	
	c.1262T>G; c.1312C>T comp het	L421R; R438*	/	NOA (1)	German	303 infertile men	(van der Bijl et al., 2019)	VUS; P	3	
	c.116+1del; c.1645_1657del comp het	splicing; H549Afs*9	/	NOA (1)	Spanish	17 men with NOA	(Krausz et al., 2020)	LP	3	
	c.962G>A hom	R321H	POI (1 sister)	NOA (1 brother)	/	/	(Jaillard et al., 2020)	LP	3	
	c.1942G>A; c.1951_1953del double hom	A648T; L652del	POI (1 sister)	NOA (2 brothers)	Iranian	/	(Akbari et al., 2022)	VUS; LP	3	
	c.2627G>A hom	G876E	POI (1)	/	Algerian	80 women with POI	(Tucker et al., 2022)	VUS	3	
RAD21L1	c.1543C>T hom	R514*	/	NOA (1)	Pakistanian	147 men with NOA	(Krausz et al., 2020)	VUS	2	AR causes NOA
REC8	c.1035_1036dup; c.624+1G>A comp het	E346Gfs*72; splicing	POI (1)	/	French	80 women with POI	(Tucker et al., 2022)	VUS; P	3	AR causes infertility in both sexes
	c.860_861del hom	P287Rfs*74	/	NOA (1)	Tunisian	96 men with NOA	(Kherraf et al., 2022)	LP	3	

Abbreviations: hom - homozygous; het - heterozygous; comp het - compound het; N. - number of individuals; MC - miscarriages; RM - recurrent miscarriages; POI - Primary ovarian insufficiency; NOA - non-obstructive azoospermia; VUS - variant of unknown significance; B - benign; LB - likely benign; P- pathogenic; LP - likely pathogenic; AD - autosomal dominant; AR - autosomal recessive. The American College of Medical Genetics and Genomics (ACMG) prediction was achieved by Varsome (https://varsome.com/) (Kopanos et al., 2019) and Franklin (https://franklin.genoox.com). Evidence level 2: moderate evidence; 3: strong evidence.

4 CONCLUSIONS AND FUTURE DIRECTIONS

Although many factors have been associated with RPL, half of the cases remain clinically unsolved. Through evaluation of the couple's clinical presentation, family history, conception characteristics, and analysis of exome sequencing variants, we have reached the conclusion that the recessive stop gain mutation in *HORMAD2* in the female partner is most likely the cause of her eight consecutive first-trimester miscarriages. The work we described on this case illustrated the complexity in pinpointing the causative gene since the patient had another variant in in another gene with a previously reported role in POI. However, the presence of MI abnormalities in one of the conceptions of our patient favored the causative role of *HORMAD2* mutation in her defect. It remains to see in the future if other women with recessive pathogenic variants in *HORMAD2* have miscarriages with or without live births.

While writing this thesis, I was also working on another project to complete the characterization of abnormalities in heterozygous and homozygous *Hfm1* knockout mice in collaboration with Prof. Teruko Taketo. Previously our lab has identified a homozygous protein-truncating mutation in two sisters with RHM and three heterozygous pathogenic or likely pathogenic protein-truncating variants, each in one patient, in three unrelated patients with RHM, RM, or/and infertility. I am performing *in vitro maturation* (IVM) and activation of *Hfm1*$^{-/-}$ oocytes and using confocal and live imaging to evaluate the impact of HFM1-deficiency on the oocyte meiotic progression.

4.1 Additional work performed during my master's degree

While working on the project described in Chapter 2, I contributed to other projects in collaboration with my colleagues during the past three years. Listed below are additional work I performed during my master's degree:

- Screened 15 patients for *NLRP7* mutation and identified pathogenic variants in 3 patients. Validated and segregated by Sanger sequencing to demonstrate their biallelic status.
- Started a list of 500 genes in ovarian functions. Validated and segregated 34 monoallelic P/LP heterozygous variants in these genes in patients who are unsolved.
- Re-sequenced the variant found in *FOXL2* in both directions to complete the data. Segregated and analyzed the consequences of 3 monoallelic pathogenic or likely pathogenic variants in other genes on splicing by RT-PCR (RNA extracted from EBV-transformed lymphoblastoid cell lines of the patients). Took histopathology images of one POC, assembled figures, and helped in the preparation of a manuscript (An Emerging Role of Meiosis I Defects in the Genesis of Androgenetic Hydatidiform Moles).
- Extracted DNA from the patient and family members, submitted sample for exome sequencing, analyzed and evaluated the pathogenicity of variants, which contributed to the manuscript in Appendix II - A report of two homozygous *TERB1* protein-truncating variants in two unrelated women with primary infertility
- Generated and cryopreserved 13 lymphoblastoid cell lines from patient blood samples.
- Extracted DNA from 64 blood and saliva samples from patients and relatives.
- Evaluated reproductive abnormality in *Hfm1*$^{+/-}$ mice at 2, 6, and 10 months by recording their estrous cyclicity. Identified that *Hfm1*$^{+/-}$ mice have significantly longer average cycle length than wildtype at 2 months.

- Currently characterizing ovarian abnormalities in *Hfm1*$^{-/-}$ mice by IVM, confocal microscopy, and live imaging.

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APPENDICES

Appendix I - List of websites

BioRender

https://www.biorender.com/

Franklin

https://franklin.genoox.com - Franklin by Genoox

gnomAD v2.1.1

https://gnomad.broadinstitute.org/

Leiden Open Variation Database

https://databases.lovd.nl/

Primer3Plus

https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

UCSC Genome Browser

https://genome.ucsc.edu/

UniProt

https://www.uniprot.org/

VarSome

https://varsome.com/

Appendix II - A report of two homozygous *TERB1* protein-truncating variants in two unrelated women with primary infertility

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GENETICS

A report of two homozygous *TERB1* protein-truncating variants in two unrelated women with primary infertility

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Abstract

Purpose To investigate the genetic etiology of patients with female infertility.

Methods Whole Exome Sequencing was performed on genomic DNA extracted from the patient's blood. Exome data were filtered for damaging rare biallelic variants in genes with possible roles in reproduction. Sanger sequencing was used to validate the selected variants and segregate them in family members.

Results A novel homozygous likely pathogenic variant, c.626G>A, p.Trp209*, was identified in the *TERB1* gene of the patient. Additionally, we report a second homozygous pathogenic *TERB1* variant, c.1703C>G, p.Ser568*, in an infertile woman whose azoospermic brother was previously described to be homozygous for her variant.

Conclusions Here, we report for the first time two homozygous likely pathogenic and pathogenic *TERB1* variants, c.626G>A, p.Trp209* and c.1703C>G, p.Ser568*, respectively, in two unrelated women with primary infertility. *TERB1* is known to play an essential role in homologous chromosome movement, synapsis, and recombination during the meiotic prophase I and has an established role in male infertility in humans. Our data add *TERB1* to the shortlist of Meiosis I genes associated with human infertility in both sexes.

Keywords *TERB1* · Female infertility · Genetics · Meiosis · Mutation

Introduction

Infertility is defined as the inability to conceive after a year of unprotected intercourse [1]. This disorder, affecting globally 15% of the general population [2], can be categorized into two groups: primary and secondary. Unlike primary infertility, secondary infertility patients have had previous successful pregnancies [3]. Due to the complex nature of human reproductive physiology, there is notable variety in how infertility is manifested. In women, ovulatory problems make up the majority of infertility cases. Among these are polycystic ovarian syndrome, premature ovarian insufficiency (POI), and hormonal irregularities caused by hypothalamic dysfunction [4]. In men, abnormalities in sperm count, morphology, motility, and function can lead to infertility [5], with up to 15% of these cases being attributable to genetic defects [6].

Over the years, the growth in access to whole-genome and exome sequencing technologies has facilitated associating many genes of the reproductive axis with human infertility [2]. Although genetic defects in various biological processes, including gonad formation, hormonal regulation, and meiosis, have been described to cause infertility, pathogenic variants in genes contributing to gamete quality and production have been highlighted to have the most severe impact on reproductive success [2]. However, despite the high frequency of infertility, improved insight into its

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biological mechanisms, and the availability of sequencing technologies, the genetic and pathophysiological heterogeneity of infertility render numerous causative genes and variants undiscovered.

Here, we report two homozygous stop-gain variants in the telomere repeat binding bouquet formation protein 1 (*TERB1*) gene in two unrelated infertile women from familial cases. So far, *TERB1* variants have only been reported in infertile men. Our findings show, for the first time, that they are also associated with primary female infertility.

Materials and methods

Clinical case reports

Case 1 The first family analyzed in this study is of Egyptian origin and includes a female (patient ID 2105) with five years of primary infertility due to diminished ovarian reserve, and her brother (patient ID 2104) with ten years of infertility with three partners due to non-obstructive azoospermia (Fig. 1a). The parents of the infertile siblings are first cousins. Clinical findings of the infertile female are summarized in Table 1.

Case 2 The second infertile female is from a previously reported family (Fig. 1b) consisting of two unaffected siblings and an infertile non-obstructive azoospermic male who is homozygous for a pathogenic protein-truncating *TERB1* variant, c.1703C > G, p.Ser568* [9]. The fertility status of the second homozygous brother is unknown. The affected sister has an unfulfilled wish to have a child for 4–5 years and had one spontaneous pregnancy that resulted in a very early miscarriage. Afterwards, medically assisted reproduction treatment did not result in pregnancy. No DNA was available from the infertile sister prior to publishing the original report.

Subjects and DNA extraction All participants of this study have provided their written informed consents. Blood samples were collected from the patients as well as family members when available. Flexigene DNA Kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from whole blood following manufacturer instructions.

Library preparation and whole exome sequence filtering Roche Nimbelgen SeqCap EZ Human Exomes or MedExomes capture kits were used to capture five hundred nanograms of peripheral blood leukocyte DNA from the patients. The DNA was sequenced with paired-end 100 base-pair reads on Illumina HiSeq 6000. Burrows-Wheeler Aligner (V.0.7.17) [10] was utilized to map the sequence reads to the human reference genome (hg19). Picard (V2.27.4) [11] was used as described previously [12] to flag duplicate reads, which were excluded from our analysis. Variant calling was completed with GATK HaplotypeCaller (V.4.2.4.0). ANNOVAR and custom scripts were used to complete variant calling as described previously [12]. The resulting annotated variants were filtered against frequent germline polymorphisms found in The 1000 Genomes Project, Genome Aggregation Database (gnomAD) (V2.1.1) [13] and dbSNP135. Next, only biallelic variants with a maximum population minor-allele frequency (MAF) of less than or equal to 0.01 were kept. Among these, variants seen in ≤ 5 individuals out of approximately 4400 in-house controls, that lead to insertion/deletion, protein-truncating, or affect conserved missense, and have a Combined Annotation Dependent Depletion (CADD) [14] score ≥ 10 were selected for. Remaining variants in genes with a potential role in reproduction were then classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines using Varsome [15], which utilizes a point system as previously described [16] to evaluate and score the pathogenic potential of the variant of interest. Only variants that are classified as pathogenic (score ≥ 10), likely pathogenic (6 ≤ score ≤ 10), or variant of uncertain significance (VUS) (0 ≤ score ≤ 5) were selected.

Mutation analyses Primers designed with Primer3Plus [17] were used to amplify target regions with the variants using PCR conditions previously described [18]. Sanger sequencing was used to validate candidate variants and segregate the validated variants among available family members. All variants detected have been submitted to the Leiden Open Variation Database (https://databases.lovd.nl/shared/individuals/00440109) (patient IDs 00446588 for case 1 and 00446590 for case 2).

Results

Case 1 To potentially identify the genetic etiology of infertility in this family, whole exome sequencing was performed on 2105 using blood DNA. Given the parents are first cousins and did not have problem conceiving, the recessive mode of inheritance of the defect was prioritized. Exome data were filtered according to the criteria described in the Materials and Methods and resulted in the identification of two genes with candidate variants: a novel homozygous stop-gain variant, NM_001136505.2:c.626G>A, p.Trp209*, in *TERB1,* and two novel multiple heterozygous nonsynonymous variants, NM_053006:c.2T>A, p.M1K and NM_053006:c.728G>T, p.C243F, in *TSSK2* (Supplementary Figs. 1 and 2). The identified variants were classified as likely

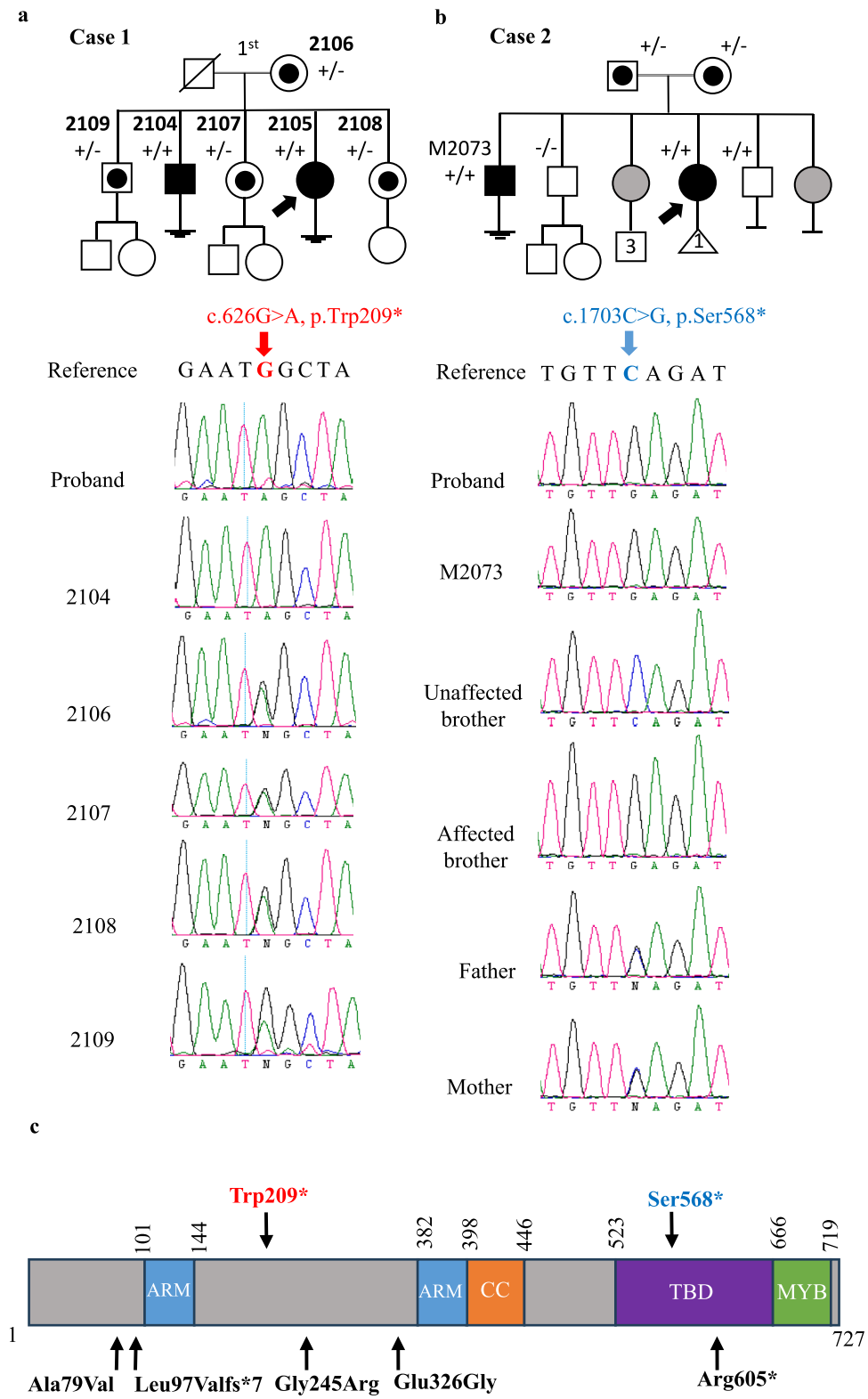

Fig. 1 Identification of the likely pathogenic and pathogenic *TERB1* variants in two families with primary female infertility. **a** The family pedigree and chromatograms for Case 1. **b** The family pedigree [9] and chromatograms for Case 2. '+/+' and '+/-' indicate family members who are homozygous and heterozygous for the variant, respectively. '-/-' indicate family members with wildtype alleles. The black filled circle and square represent the infertile female and azoospermic male, respectively. Grey circles indicate genetically not tested individuals. Symbols with the black-dot represent the heterozygous carriers. Black arrow indicates the proband. Triangle represents miscarriage. The fertility status of M2073's homozygous brother is unknown. **c** Schematic representation of the human *TERB1* protein and its functional domains. Interdomains are indicated in grey, and the domains are colored. ARM, armadillo repeats; CC, coiled-coil domain; TBD, TRF-1 binding domain; MYB, Myb-like domain. Mutated amino acids above the protein structure are the *TERB1* variants found in our patients (red: novel, blue: reported in [9]), while those below the protein structure in black are the *TERB1* variants reported in previous literature

pathogenic, likely pathogenic, and likely benign, respectively [15]. Sanger sequencing was used to validate and segregate the candidate variants, which led to the confirmation of the *TERB1* variant (Fig. 1a) and the exclusion of the *TSSK2* variants since c.2T>A, p.M1K was not validated in the patient (Supplementary Figs. 1 and 2).

Table 1 Clinical data of patient 2105

Parameter	Measurement
Age at menarche	13
Menstrual cycle	30 days, lasting 3–5 days
Luteinizing Hormone levels	Elevated (18.5 IU/L)
Follicle Stimulating Hormone levels	Elevated (22.3 IU/L)
Anti-Mullerian Hormone levels	Severely decreased (0.3 ng/mL)
Ovary size	Small ovaries Right → 1,96 × 1,70 × 1,52 Left → 2,26 × 1,07 × 1,51
Partner sperm parameters	Normal andrological parameters Ejaculate volume = 2 mL Sperm count = 60 million/ejaculate Sperm motility = 85% Progressive motility = 54% Morphology index = 40%

The clinical data was acquired on day 2 of menses when the patient was 26 years and 8 months old. The reference level of the hormones at follicular phase are the following: Luteinizing Hormone = 1–12 IU/L [7], Follicle Stimulating Hormone = 1–9 IU/L [7], Anti-Mullerian Hormone = 2–6.8 ng/mL [8].

The *TERB1* variant is located in a run of homozygosity of 11.1-Mb on chromosome 16 and was predicted by the R package *masonmd* (Make Sense of nonsense-mediated decay (NMD)) [19] and Mutation Taster [20] to trigger NMD. Segregation analysis showed that this variant is also homozygous in the affected azoospermic brother, while the three unaffected siblings and parents are all heterozygous carriers (Fig. 1a). To exclude the presence of any other recessive causative variants responsible for the infertility of the azoospermic brother, we next performed exome sequencing on his blood DNA and filtered the exome data under the same criteria described above. Our analysis did not reveal any other candidate gene with plausible recessive variants that may explain the phenotype of the infertile brother (Supplementary Fig. 1).

TERB1 is involved in the pairing of homologous chromosomes during meiotic prophase I [21] and has an established role in the causation of male infertility due to meiotic arrest and consequently azoospermia (Table 2). Based on its function, the phenotype of the affected brother, and the pathogenicity classification of the variant, we conclude that the novel homozygous likely pathogenic variant, c.626G>A, p.Trp209*, in *TERB1* is the most plausible candidate to explain the infertility of the two siblings.

Case 2 DNA analysis of the infertile sister revealed that she is also homozygous for the *TERB1* variant found in her brother, NM_001136505.2:c.1703C>G, p.Ser568* (Fig. 1b). The variant was also predicted by *masonmd* [19] and Mutation Taster [20] to trigger NMD.

Discussion

In this paper, we report a novel likely pathogenic protein-truncating variant, p.Trp209*, in the *TERB1* gene of an infertile woman and her azoospermic brother. We also report a second infertile female, from a previously described family [9], who is homozygous for another pathogenic protein-truncating variant in *TERB1*.

TERB1 encodes for a 727 amino acid nuclear protein consisting of two ARM, one coiled-coil, one TERF1-interacting, and one Myb domains (Fig. 1c) [21]. TERB1 plays a critical role in the attachment of telomeres to the nuclear envelope and is required for homologous chromosome movement, pairing, synapsis, and recombination. It interacts with the telomeric repeat binding factor 1 (TERF1 in humans and *trf1* in mice) and mediates the assembly of the meiotic telomere complex (MTC) [25], which includes

Table 2 Previously reported recessive *TERB1* variants

Reference	Patient ID	Variant*	Zygosity	ACMG Classification by Varsome	Phenotype
Kherraf et al., 2022 [22]	P0145	c.733G>A, p.Gly245Arg	Hom	VUS (4P: 0B)	NOA
Salas-Huetos et al., 2021 [9]	Individual 2	c.977A>G, p.Glu326Gly	Hom	VUS (1P: 1B)	NOA
	M2073**	c.1703C>G, p.Ser568*	Hom	Pathogenic	NOA
Krausz et al., 2020 [23]	M468	c.236C>T, p. Ala79Val	Hom	VUS (2P: 0B)	NOA
	10–200 & brother	c.289_290del, p. Leu97Valfs*7	Multiple Het	Pathogenic	NOA
		c.1813C>T, p. Arg605*		Pathogenic	NOA
Alhathal et al., 2020 [24]	19DG1792	c.733G>A, p.Gly245Arg	Hom	VUS (4P: 0B)	NOA
	19DG1816		Hom		NOA

*All variants are provided in NM_001136505.2. **Previously reported male proband whose infertile sister is reported in this study. 'Hom' and 'Multiple Het' are short for homozygous and multiple heterozygous, respectively. 'VUS' is for variant of uncertain significance, 'P' for pathogenic points, and 'B' for benign points.'NOA' stands for non-obstructive azoospermia.

another TERB protein, TERB2, and membrane-anchored junction protein (MAJIN) [26], a transmembrane protein of the inner nuclear membrane. The MTC localizes to the nucleus and anchors the chromosomes to SUN domain-containing protein 1 (SUN1), another transmembrane protein of the inner nuclear membrane, and KASH domain-containing protein 5 (KASH5), a transmembrane protein of the outer nuclear membrane [27]. SUN1 and KASH5 form the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex and attach the MTC to the Dynein-Dynactin complex in the cytoplasm [28]. Altogether, these interacting proteins between the chromosomes in the nucleus and the cytoplasmic cytoskeleton ensure the rapid movement of homologous chromosomes for pairing, synapsis, and recombination [29].

In mice, deleterious mutations in any of *Terb1* [26], *Terb2* [26], *Majin* [26], *Sun1* [30], or *Kash5* [31] result in the loss of germ cells, gonadal dysgenesis, and infertility in both sexes. In humans, biallelic variants in *TERB1* [9, 22–24], *TERB2* [9], *MAJIN* [9], *SUN1* [32], and *KASH5* [32] have been shown to cause non-obstructive azoospermia. However, of these five genes, only biallelic variants in *KASH5* have so far been reported in infertile women (Supplementary Table 1). Some of these women had primary infertility due to POI [33, 34] while others had diminished ovarian reserve and/or recurrent miscarriage [35].

In addition to the members of the MTC and LINC complexes, recessive defects in approximately 50 Meiosis I genes have been shown to cause infertility in both male and female mice [36]. However, to our knowledge, biallelic variants in about 20 Meiosis I genes have been reported to cause male and female infertility in humans (summarized in Supplementary Table 1). Results from both mice and human studies have demonstrated a consistent difference in the severity of the phenotype between the two sexes. While in men, deleterious variants cause mostly a complete arrest of Meiosis I and lead to non-obstructive azoospermia, in women, the meiotic arrest appears to be partial and results in a spectrum of reproductive outcomes, ranging from infertility to POI, diminished ovarian reserve, early embryonic arrest after medically assisted reproduction, recurrent molar pregnancy and/or miscarriage (Supplementary Table 1). This sexual dimorphism was described a long time ago in mice [36, 37] and humans [38]. However, due to the phenotype-based nature of human studies, this spectrum of diverse reproductive outcomes seen in women adds another layer of complexity to the highly heterogeneous entity of female infertility, consequently, hampering and delaying the identification of its causative genes and associated variants.

Herein, we describe, for the first time, the association of recessive pathogenic *TERB1* variants with primary female infertility in two unrelated families. Our report adds *TERB1* to the, as of yet, short list of Meiosis I genes associated with human infertility in both sexes.

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Declarations

Conflict of interest The authors have declared that no conflict of interest exists.

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