IDENTIFICATION OF NEW GENES FOR RECURRENT HYDATIDIFORM MOLES

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Dear future Maryam

Hopefully when at some point in future you come to read this thesis again, you will be happy with what you have done and learned through these six years of PhD and remember the passion and joy you felt when you saw the growth of the cells under the microscope or learned new techniques such as ddPCR with BioRad team and you could not wait to show them your nice results! I also hope you implement the lessons you got from both good times and bad times during your PhD, in your future life. Don't forget that you LEARNED, and you LOVED it.

Abstract

Hydatidiform mole (HM) is a rare complication of pregnancy characterized by excessive proliferation of the trophoblast and abnormal embryonic development. The common form of HM is sporadic, non-recurrent, and affects 1 in every 600 pregnancies. Among women with one mole, 1–4% will develop a second mole, which is referred as recurrent hydatidiform moles (RHM), and 10–25% will experience a second reproductive loss, mostly as a miscarriage (MC). When I started my PhD project in 2017, bi-allelic mutations in two maternal-effect genes, *NLRP7* and *KHDC3L*, were known to underlie the causation of RHM. NLRP7 and KHDC3L are members of subcortical maternal complex (SCMC), a proteinaceous complex in mammalian oocytes and preimplantation embryos, required for female fertility and early embryogenesis. Bi-allelic mutations in these two genes were associated with recurrent diploid biparental HMs (one chromosome set from each parent) (1).

NLRP7 and *KHDC3L* explain 60% of RHM cases; however, still, there was a question, "what are the causative genes for the remaining 40% of patients without mutations in these two genes?". In 2018, bi-allelic mutations in *PADI6* which is another important SCMC component were found to underlie the phenotype of a patient with one HM and four miscarriages. In this study, my contribution was the demonstration of the colocalization of PADI6 with NLRP7 at the cortex of human oocyte and preimplantation embryos (2). In the same year, my laboratory demonstrated that bi-allelic mutations in three meiotic genes *MEI1*, *TOP6BL* and *REC114* (3), underlie the genetic etiology of androgenetic RHMs (both chromosome sets are paternally derived), miscarriages, and infertility.

Chapters 2,3 and 4 describe the work of gene identification in patients with recurrent HMs and miscarriages. To identify additional novel genes responsible for this entity, our approach was to screen new patients for mutations in *NLRP7* and *KHDC3L* and perform whole exome sequencing (WES) on patients who are negative, analyze their exome data, and validate identified variants in candidate genes. The main challenge of this work was the high genetic heterogeneity of patients with RHM, since we were not able to find any two patients with mutations in the same gene.

Chapter 2 contains two manuscripts. In the first, I identified the first patient with RHM and a bi-allelic *NLRP5* mutation, and a second patient with recurrent HM and miscarriages with a homozygous *PADI6* mutation and five novel mutations in *NLRP7*. The patient with *PADI6* had four molar pregnancies, two of which had fetuses with various abnormalities including placental mesenchymal dysplasia and intra-uterine growth restriction, which are features of Beckwith-Wiedemann syndrome (BWS) and Silver Russell syndrome (SRS), respectively. All three genes are members of the SCMC. This study highlighted a continuous spectrum of abnormalities associated with deficiencies in the SCMC genes from primary infertility to live birth with imprinting disorders, which all originate from a defective oocyte.

In the second manuscript which describes the genetics of RHM in Mexico, I did some mutational screening in *NLRP7* and haplotype analysis of all the SNPs covered by *NLRP7* Sanger sequencing and demonstrated the inheritance of a founder mutation, L750V, in the Mexican population on a shared haplotype by patients from various Mexican states.

Chapter 3 describes the coincidental identification of a second unrelated patient with bi-allelic mutation in *CCNB3*, a novel gene responsible for recurrent miscarriage (4) in a patient with 16

miscarriages. In this manuscript, we also characterized the genotype of one of the patient's miscarriages and found its triploid digynic genotype due to the failure of meiosis I (5).

In chapter 4, we identified bi-allelic protein truncating mutations in three genes, *HFM1*, *MAJIN*, *SYCP2*, a compound heterozygous variant in *TOPBP1* (all the four genes have roles in meiosis I) and a homozygous missense in *FOXL2*, which is a transcription factor essential for proper reproductive function in females. *HFM1* mutation was found in a familial case consisting of two sisters affected with RHM. At least one molar tissues from the patients with *MAJIN*, *HFM1*, *FOXL2*, *TOPBP1* mutations were found to be androgenetic monospermic. The molar tissues from the patient with *SYCP2* mutations were reported to us as complete moles. Functionally, *HFM1*, *MAJIN*, *SYCP2* and *TOPBP1* are required during meiosis prophase I for homologous chromosomes pairing and recombination. *FOXL2* is essential for proper reproductive function in females and defects in this gene causes premature ovarian insufficiency (POI). Except for *FOXL2*, mutations in all the other genes are associated with recurrent androgenetic moles suggesting that various defects in female meiosis I are responsible for androgenetic moles.

RÉSUMÉ

La môle hydatiforme (MH) est une complication rare de la grossesse caractérisée par une prolifération excessive du trophoblaste et un développement embryonnaire anormal. La forme courante des MH est sporadique, non récurrente et affecte 1 grossesse sur 600. Parmi les femmes ayant eu une grossesse môlaire, 1 à 4 % développeront une deuxième môle, et donc appelée môle hydatiforme récurrente (MHR). Dix à 25 % des femmes ayant eu une grossesse môlaire subiront une deuxième perte de grossesse, principalement sous la forme de fausse couche (MC). Lorsque j'ai commencé mon projet de doctorat en Janiver 2017, des mutations bi-alléliques dans deux gènes à effet maternel, NLRP7 et KHDC3L, étaient connues pour être à l'origine des MHR. NLRP7 et KHDC3L sont membres d'un complexe protéique situé dans la région sous-cortical (SCMC) de l'ovocyte et les embryons préimplantatoires des mammifères. Ce complexe est nécessaire à la fertilité féminine et à l'embryogenèse précoce. Des mutations bi-alléliques dans ces deux gènes ont été associées à des MHR dont le genome est diploïde biparentale (un jeu de chromosomes de chaque parent) (1).

NLRP7 et *KHDC3L* expliquent 60 % des cas de MHR; cependant, il y avait toujours une question, "quels sont les gènes responsables pour les 40% restantes de patientes sans mutations dans ces deux gènes?" En 2018, des mutations bi-alléliques dans PADI6, qui est un autre membre du SCMC, se sont avérées être la cause d'une grossesse môlaire et quatre fausses couches chez une patiente. Dans cette étude, ma contribution a été la démonstration de la co-localisation de PADI6 avec NLRP7 au niveau du cortex des ovocytes humains et d'embryons préimplantatoires (2). La même année, mon laboratoire a démontré que des mutations bi-alléliques dans trois gènes méiotiques *MEI1*, *TOP6BL* et *REC114* (3) expliquent l'étiologie

génétique des MHR androgénétiques (les deux ensembles de chromosomes sont d'origine paternelle), des fausses couches et de l'infertilité.

Les chapitres 2, 3 et 4 décrivent le travail d'identification des gènes chez les patientes présentant des MHR et des fausses couches. Pour ce faire, notre approche consistait à dépister les mutations de nouveaux patientes dans *NLRP7* et *KHDC3L* et à effectuer un séquençage complet de l'exome (WES) sur les patientes négatives, à analyser leurs données d'exomes et à valider les variantes identifiées dans les gènes candidats. Le principal défi de ce travail était la grande hétérogénéité génétique des patientes atteintes de MHR, puisque nous n'avons pas été en mesure de trouver deux patientes présentant des mutations dans le même gène.

Le chapitre 2 contient deux manuscrits. Dans le premier, j'ai identifié la premiere patiente avec MHR et une mutation bi-allélique dans le gène *NLRP5*, et une deuxième patiente avec MHR et fausses couches avec une mutation *PADI6* à l'état homozygote et cinq nouvelles mutations dans *NLRP7*. La patiente atteinte dans *PADI6* a eu quatre grossesses molaires, dont deux avaient des fœtus présentant diverses anomalies, notamment une dysplasie mésenchymateuse placentaire et une restriction de croissance intra-utérine, caractéristiques du syndrome de Beckwith-Wiedemann (BWS) et du syndrome de Silver Russell (SRS), respectivement. Les trois gènes sont membres du SCMC. Cette étude a mis en évidence un spectre continu d'anomalies associées à des déficiences dans les gènes SCMC de l'infertilité primaire à la naissance vivante avec troubles de l'empreinte génétique, qui proviennent toutes d'un ovocyte défectueux.

Dans le deuxième manuscrit qui décrit la génétique de MHR au Mexique, j'ai effectué un dépistage mutationnel dans *NLRP7* et une analyse d'haplotype de tous les SNP couverts par le

séquençage NLRP7 Sanger et démontré la transmission de la mutation fondatrice, L750V, dans la population mexicaine sur un haplotype commun a des patientes de divers états mexicains.

Le chapitre 3 décrit l'identification fortuite d'une deuxième patiente avec une mutation bi-allélique dans le gène *CCNB3*, un nouveau gène récemment identife comee responsable de fausses couches récurrentes (4) chez un patient ayant fait 16 fausses couches. Dans ce manuscrit, nous avons également caractérisé le génotype d'une des fausses couches de la patiente et trouvé son génotype triploïde digenique dû à l'échec de la méiose I (5).

Dans le chapitre 4, nous avons identifié des mutations bi-alléliques qui entrainent la troncation des protéines codees par trois gènes, *HFM1*, *MAJIN*, *SYCP2*, deux variants faux-sens sur les deux allèles du gène TOPBP*1* (ayant des rôles dans la méiose I) et un faux-sens à l'état homozygote dans *FOXL2*, qui est un facteur de transcription essentiel au bon fonctionnement des ovaires chez les femelles. La mutation *HFM1* a été trouvée dans un cas familial composé de deux sœurs atteintes de MHR. Au moins, un tissu molaire des patientes porteuses de mutations *MAJIN*, *HFM1*, *FOXL2*, *TOPBP1* s'est avéré androgénétique monospermique. Les tissus molaires de la patiente porteuse de mutations *SYCP2* nous ont été rapportés comme des MH complètes. Fonctionnellement, *HFM1*, *MAJIN*, *SYCP2* et *TOPBP1* sont nécessaires pendant la prophase I de la méiose pour l'appariement et la recombinaison des chromosomes homologues. *FOXL2* est essentiel au bon fonctionnement des ovaires et les défauts de ce gène provoquent une insuffisance ovarienne prématurée (POI). À l'exception de *FOXL2*, des mutations dans tous les autres gènes sont associées à des MH androgénétiques récurrentes suggérant que divers défauts de la méiose I chez la femme sont responsables de moles androgenetiques.

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LIST OF ABBREVIATIONS

AnCHM	Androgenetic CHM
CDKN1C	Cyclin dependent kinase inhibitor 1C
CHM	Complete hydatidiform mole
CV	Chorionic villous
MEG	Maternal effect gene
DMR	Differentially methylated regions
DPPA5	Developmental Pluripotency Associated 5
FFPE	Formalin-fixed, paraffin-embedded
FISH	Fluorescent in situ hybridization
FOXL2	Forkhead box transcription factor
GTN	Gestational trophoblastic neoplasia
GV	Germinal vesicles
H&E	Hematoxylin and eosin
hCG	Human chorionic gonadotropin
HEK293	Human Embryonic Kidney 293 cells
hESCs	Human embryonic stem cells
HM	Hydatidiform mole
HFM1	Helicase for meiosis 1
ICSI	Intra-cytoplamsic sperm injection
Il1r1	Type 1 IL-1 receptor

IL-1β	Interleukin-1 beta
IVF	In-vitro fertilization
KHDC1	K homology domain containing 1
Khdc3	KH domain-containing protein 3
KHDC3L	KH domain containing 3 like, subcortical maternal complex member
LCL	Lymphoblastoid cell lines
LRR	Leucine Rich Repeat
MAF	Minor allele frequency
MC	Miscarriages
MEI1	Meiotic double-stranded break formation protein 1
MAJIN	Membrane-anchored junction protein
MII	Metaphase II
NACHT	Domain present in NAIP, CIITA, HET-E, TP-1
NLRP2	NLR family pyrin domain containing 2
NLRP5	NLR family pyrin domain containing 5
NLRP7	NLR family pyrin domain containing 7
NOD	Nucleotide oligomerization domain
NP	Normal pregnancy
NRBC	Nucleated red blood cell
NSV	Non-synonymous variant
OOEP	Oocyte expressed protein

Padi6	Peptidyl arginine deiminase 6
PB	Polar body
PBMC	Peripheral blood mononuclear cells
PGD	Preimplantation genetic diagnosis
PHM	Partial hydatidiform mole
POC	Products of conception
NM	Non molar
PTD	Persistent trophoblastic disease
REC114	REC114 meiotic recombination protein
RHM	Recurrent hydatidiform moles
RM	Recurrent miscarriages
SA	Spontaneous abortion
SB	Stillbirth
SNP	Single nucleotide polymorphisms
SCMC	Subcortical maternal complex (SCMC)
SYCP2	Synaptonemal complex protein 2
THP1	Monocytic cell line
TLE6	TLE family member 6, subcortical maternal complex member
TOP6BL(C11orf80)	Type 2 DNA topoisomerase 6 subunit B-like
TOPBP1	DNA Topoisomerase II-binding protein 1
WES	Whole exome sequencing

ZP	Zona pellucida

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FORMAT OF THE THESIS

The thesis is a manuscript-based format, which conforms to the Graduate and Postdoctoral Studies Guidelines for Thesis Preparation at McGill University. The studies described here were performed under the supervision of Dr. Rima Slim. This thesis is comprised of five chapters, of which two chapters are in the form of manuscripts that were published. Chapter 1 is a literature review and general introduction that recapitulates the various aspects of the pathology of hydatidiform moles and relevant background to this thesis. Chapter 2 contains two manuscripts that were published in the Journal of Clinical Genetics, 2021 (PMID: 33583041) and Journal of Assisted Reproduction and Genetics in 2021 (PMID: 33751332). Chapter 3 is a manuscript that was published in the Journal of Medical Genetics in 2021 (PMID: 34021051). Chapter 4 is a manuscript in preparation. Prefaces for chapters 2, 3 and 4 are used to connect between the chapters and to ensure the continuity of the thesis. Chapter 5 includes a global discussion of the findings from this thesis in greater detail, alongside possible future experiments. The references of all the chapters are provided at the end of the thesis. The annex at the end of the thesis contains the permission of the journals for the three publications and the certification of ethical acceptability for research involving human subjects.

CONTRIBUTION OF AUTHORS

Chapter 2 (first paper): Novel pathogenic variants in *NLRP7, NLRP5* and *PADI6* in patients with recurrent hydatidiform moles and reproductive failure

Maryam Rezaei, Beena Suresh, Eric Bereke, Zahra Hadipour, Monica Aguinaga, JianHua Qian, Rashmi Bagga, Majid Fardaei, Reda Hemida, Sujatha Jagadeesh, Jacek Majewski, Rima Slim

MR performed DNA preparation, mutational screening, genotyping, and contributed to the writing of the manuscript. EB and JM performed exome data analysis. BS, MA, MF, RH, SJ, RB referred patients, retrieved archived tissues were appropriate and recapitulated clinical data. RS supervised the work, performed morphological analysis, coordinated and analyzed p57 immunohistochemistry, provided financial support, and wrote the manuscript.

Chapter 2 (second paper): The genetics of recurrent hydatidiform moles in Mexico: further evidence of a strong founder effect for one mutation in *NLRP7* and its widespread

Mónica Aguinaga, Maryam Rezaei, Irma Monroy, Nawel Mechtouf, Javier Pérez, Elsa Moreno, Yolotzin Valdespino, Carolina Galaz, Guadalupe Razo, Daniela Medina, Raúl Piña, and Rima Slim

MA performed data collection, designed the study, wrote the first draft of the manuscript, and contributed to the final version. MR performed mutation analysis on some patients, characterized one large deletion, and analyzed the haplotype containing the founder mutation. NM genotyped molar tissues and performed mutation analysis on some patients and molar tissues; IM, MN, JP, EM, YV, CG, GR referred patients and provided follow-up clinical information. RS designed and supervised the study, provided financial support, and contributed to the writing of the manuscript.

Chapter 3: A protein-truncating mutation in *CCNB3* in a patient with recurrent miscarriages and failure of meiosis I

Maryam Rezaei, William Buckett, Eric Bareke, Urvashi Surti, Jacek Majewski, Rima Slim

MR performed DNA preparation, validated the causative variant, performed RT-PCR, genotyped of the archived tissue from the conception and contributed to the writing of the manuscript. WB referred the patient. EB and JM performed exome data analysis. US performed the FISH analysis. RS supervised the work, provided financial support and wrote the manuscript.

Chapter 4: Manuscript in preparation reporting the identification of new genes for recurrent hydatidiform moles

Maryam Rezaei performed some DNA preparation and submission to Genome Quebec, exome data filtering, identified causative mutations in two of the novel genes, and wrote the draft of the manuscript that is included in this chapter. Another member of the lab, Iqbaljit Kaur Sandhu, performed tissue sections and H&E staining and genotyped some molar tissues. Eric Bareke and Jacek Majewski processed Fastq files and performed the variant calls for exome sequences. Rima Slim identified the remaining causative mutations, coordinated the analysis of p57 immunohistochemistry, SNP microarray, and FISH.

CHAPTER 1-LITERATURE REVIEW

1.1. Hydatidiform mole

1.1.1. The history of hydatidiform mole

Hydatidiform mole (HM) consists of two words; “hydatisia” a Greek word that means a drop of water and “mola” a Latin word which means millstone/false conception and indicates a mass in a woman’s womb. In 1638, Governor Winthrop in New England described HM as “innumerable distinct bodies in the form of a globe” which is the appearance of hydropic chorionic villi observed in these pregnancies. There are other terms that were used in the past to describe this unfortunate event of pregnancy such as “Monstrous birth”, "several lumps of man's seed without any alteration”, “ova that had not been impregnated”. Also, in the past, it was believed that moles were due to parasites until in 1827 when Madame Boivin recognized the chorionic origin of molar vesicles (Figure1A, B) (6). HM is characterized by the absence of, or abnormal, embryonic development and excessive proliferation of the trophoblast.

1.1.2. Epidemiology

When a hydatidiform mole occurs once, it is known as sporadic HM, which are relatively common, with a variable incidence in different ethnic groups. The incidence of HM is 1 in 600-1000 pregnancies in Western countries (7) but 2-10 times higher in Asian, African, and Latin American countries, with the highest frequency being 1 in 80 pregnancies in Indonesia (8,9).

Recurrent HM (RHM) is defined when HM happens more than once in a patient, and it is called familial recurrent HMs if it happens in more than one family member. Among women with one HM, 10-20% have other forms of reproductive loss, mainly as spontaneous abortions (1). The frequency of two miscarriages in the general population is 2–5%, while the frequency of at least one miscarriage in a patient with a prior HM is 10–20%, which is 2–4 times higher. Therefore, it

is believed that patients with an HM and miscarriages have a genetic predisposition to recurrent reproductive loss. Recurrence of a second HM varies among populations and countries and affects 1-9% of women with a prior HM (1-2 % in western countries to 2.5 - 9.4 % in Middle and Far East). A study from the United Kingdom and Wales between 1973-1983 found that the incidence of experiencing a second HM is 1 in 76 and that of a third HM is 1 in 6.5, respectively (10). The incidence of HM is higher in women at the extremes of reproductive age. There is a relatively modest increased risk for teenagers (age <18), but with risks ranging from 1% for those aged 45 to 17% for those ≥ 50 (7,11).

1.1.3. Clinical manifestation of HM and management

Irregular vaginal bleeding in 80-90% of patients during the first trimester of gestation is the most common clinical presentation of HM. The other classic clinical symptoms may comprise hyperemesis (severe vomiting), hyperthyroidism, toxemia (high blood pressure, albuminuria, edema), pulmonary embolism and preeclampsia (pregnancy-induced hypertension) (12). Upon physical examination, clinicians detect an excessive ovarian bilateral and uterine enlargement in nearly 30% and 50% of the patients, respectively. To diagnose HM, clinicians rely on ultrasonography, which reveals the presence of echogenic structures in the placenta, the absence of a gestational sac, and/or the absence of fetal heart activity (13). These initial ultrasound observations are followed by a blood test of the human chorionic gonadotropin (hCG) which is much higher (sometime over 100,000 mIU/ml) in women with molar pregnancies than in women with normal pregnancies of matching gestational stage (14).

After suspecting a molar pregnancy, the clinician interrupts and evacuates the abnormal conception by dilatation suction and curettage (D&C) which can cure almost eighty percent of women with HM. After evacuation of the molar tissue, the patients are followed-up and

monitored on a regular basis (every week) to ensure that the levels of hCG in blood keep decreasing until they reach normal, non-pregnant level (15).

1.1.4. Histopathology-based classification of HMs

At the histopathological level, HMs are classified into two types, complete HM (CHM) and partial HM (PHM), based on the degree of trophoblastic proliferation and embryonic tissue differentiation. CHM usually have marked circumferential trophoblastic proliferation (Figure 1C) with absence of embryonic tissues of inner cell mass origin and extraembryonic membranes (chorion and amnion), while PHM have moderate trophoblastic proliferation and may contain embryonic tissues and extraembryonic membranes (16).

Epidemiological studies have shown some risk factors that could predispose to sporadic HM such as: maternal age, reproductive history (a history of miscarriages), ethnicity, and various environmental factors including diet, oral contraception, herbicides, and ionizing radiation. Among these risk factors, maternal age and a history of miscarriages are the strongest risk factors and have been replicated in several studies and populations (10).

1.1.5. Genotype-based classification of HMs

At the genotypic level, there are 4 types of HM, diploid androgenetic monospermic, diploid androgenetic dispermic, triploid dispermic, and diploid biparental. CHMs are usually diploid androgenetic with two copies of the paternal genome and no maternal genome and may originate from monospermic (two identical copies of a haploid paternal genome) fertilization in 85% of the cases or dispermic (two different haploid paternal genomes) fertilization in 15% of the cases. 98% of PHM are triploid dispermic with two copies of the paternal genome and one copy of the

maternal genome and 2% are monospermic (17–20). A minority of HM are diploid biparental with one chromosome set from each parent (Figure 1.2)

1.1.6. Androgenetic HM formation

In 1977, Kajii and Ohama et al, reported for the first time that the karyotype of common HMs are predominantly 46,XX solely paternally derived; therefore androgenetic origin of HM was proposed. They also proposed an "empty oocyte" as the mechanism for androgenesis in which an oocyte without nucleus (being either absent or inactivated) is fertilized by one sperm (monospermy) or two sperm (dispermy). In these diploid androgenetic moles, the male genomes become diploid by a haploid sperm followed by duplication of its chromosomes or through fertilization by two sperms (dispermy), or by a diploid sperm (21). One year later in another study by Wake et al, the hypothesis of empty oocytes was repeated in which after fertilization, the maternal chromosome set is eliminated or inactivated (22). However, until now, there was no scientific evidence to support the existence of empty oocyte proposal.

Another hypothesis about the possible mechanism of androgenetic HM was formulated by Golubovsky et al, who proposed that postzygotic diploidization of triploids (PDT) rather than the 'empty' oocyte concept may be at the origin of androgenetic HM because of the predominant dispermic triploid zygotes after assisted reproductive technologies. Moreover, with the lack of evidence for empty oocytes and the high frequency of triploidy observed in human reproduction failure, Golbovsky's model fits well. According to his PDT model, post-fertilization errors of triploid zygotes may explain various types of abnormal genotypic patterns observed in human conceptions. In this model, the oocyte is nucleated, not empty, and is fertilized by 2 different spermatozoa and the outcomes are i) in around 25% of the cases, the zygotes may be maintained as triploid dispermic conceptions, which leads to a partial HM, ii) in 14-32% of the cases, one

haploid genome is excluded in the first cleavage division, resulting in 2n diploid, 2n/3n mosaics and 1n/2n derivatives, and iii) in 50-60% of zygotes, a tripolar spindle is formed at the first cleavage resulting in dramatic abnormalities in chromosome distribution. In 2n/3n and 1n/2n mixoploidy, some 2n cell derivatives may develop as an HM (23). Therefore, this model provides a natural explanation for the regular appearance of 2n homozygous androgenetic moles, various mosaic/chimeric conceptions containing HM and molar/twin (24).

There are no experimental evidence demonstrating the mechanism of androgenetic HM formation during the preimplantation cleavage stages in humans. Moreover, human oocytes and embryos are scarce materials and there are challenges in working with these materials. Androgenesis has not been described in any other mammalian species. Androgenesis has been reported in at least three types of organisms such as several species of freshwater clams (Corbicula), one plant species (Cupressus dupreziana) and in stick insects (Bacillus) with diploid sperm, diploid pollen and fusion of haploid sperm pronuclei in egg as the source of diploidy in their androgenesis, respectively (25). In Drosophila melanogaster, androgenesis sometimes occurs in a line that carries a mutation affecting chromosome disjunction during cell division. The offspring are produced by doubling the ploidy of a haploid cell produced by meiosis which results in homozygosity at all loci, and all offspring are female (because YY embryos are not viable) (26).

1.1.1.7. Methods to determine the parental contribution to the HM genome

p57 immunohistochemistry (IHC) is one of the main ancillary techniques for subtyping HMs as CHM and PHM and distinguishing HMs from non-molar (NMs) specimens. p57KIP2 is a cyclin dependent kinase inhibitor encoded by the *CDKN1C* gene in the nuclei of various cells, which is regulated by imprinting (paternally imprinted, maternally expressed). This marker is used

routinely in the clinical diagnosis of HM to detect the presence of the maternal genome and consequently distinguish between partial and complete moles. p57KIP2 immunostaining is interpreted as negative when endometrial and/or extravillous trophoblastic cells (EVT) (internal positive control), exhibit nuclear p57KIP2 staining but villous stromal and/or cytotrophoblastic cells do not. When cytotrophoblast and/or villous stromal cells show nuclear staining of p57KIP2, the staining is considered as positive. With rare exceptions, CHMs are p57-negative and androgenetic diploid; partial hydatidiform moles are p57-positive and diandric triploid; and nonmolar specimens are p57-positive and biparental diploid (16,18,27).

P57 IHC can identify CHMs (androgenetic diploidy) from PHMs since CHMs lack the maternal genome but cannot distinguish PHMs (diandric monogynic triploidy) from non-molar NMs (biparental diploidy). Short tandem repeat (STR) genotyping can identify the parental source of polymorphic alleles and thus distinguish androgenetic diploidy, diandric triploidy, and biparental diploidy, which allows for specific diagnosis of CHM, PHM, and NM respectively (18).

1.2. Genetics of RHMs

The genetic causes of RHMs were unknown until 1999, a maternal recessive locus was mapped to the telomeric region of chromosome 19q in a 15.2 cM genetic interval flanked by D19S924 and D19S890. The mapping was performed using a combination of linkage search through the genome and homozygosity analysis on two families (Lebanese and German) with RHMs (28). Seven years later, in 2006, the hydatidiform mole candidate region was fine-mapped to 0.65 Mb on 19q13.4 and *NLRP7* was identified as the defective gene responsible for RHMs by

screening candidate genes in the critical interval for mutations in the original two families and two additional ones (29).

1.2.1 *NLRP7*

NLRP7 (**N**ucleotide-binding, **L**eucine-rich **R**epeat, **P**yrin domain containing 7) is the first maternal-effect gene identified as the cause for RHMs. Studies from various groups and populations concur that *NLRP7* is the major gene for RHMs and is mutated in 55% of patients Figure 1.2. All the RHMs due to mutation in *NLRP7* are diploid biparental (1) with the exception of two moles that were found to be triploid digynic in two unrelated cases (30,31)

NLRP7 protein (1037 amino acids) comprises four functional domains, the N-terminal effector domain PYRIN (PYD), a central NACHT domain for initiating oligomerization by binding ATP (which is found in NAIP, CIITA, HET-E and TP1), a NACHT-associated domain (NAD) and a C-terminal leucin rich repeat region (LRRs) (Figure 1). NLRP7 is a member of the NLR family of proteins with role in inflammation and apoptosis.

To date, approximately, 86 different bi-allelic mutations (including missense, stop codons, less than 20-bp deletions or insertions, splice mutations, and Alu mediated complex rearrangements including large deletions or insertions) in *NLRP7* have been reported and recorded in Infevers (https://infevers.umai-montpellier.fr/web/), which is an online registry of autoinflammatory disorders mutations.

In addition to these mutations, single heterozygous variants including two protein-truncating mutations, a stop codon, L823X, and approximately 17 missenses have also been reported in patients with recurrent and sporadic moles. While homozygous and compound-heterozygous variants in *NLRP7* cause RHM and associated reproductive wastage, heterozygous *NLRP7*

variants are not causative for RHM but their carriers appear to be at increased risk for reproductive wastage including sporadic moles (32). Additionally, in another study by Zhang et al. it was reported that Chinese patients with gestational trophoblastic diseases (GTD) have a higher burden of single heterozygous variants. In this study they suggested that these single non-synonymous variants (NSVs) may contribute to the genetic susceptibility for GTDs in China (33). It has also been observed that the offspring of the patients with heterozygous NLRP7 variants have aberrant methylation patterns at imprinted loci (32,34,35). Taken together, these observations indicate that patients with single heterozygous variants in *NLRP7* are at higher risk of pregnancy complications and prenatal mortality.

NLRP7 transcripts have been observed in several human tissues including uterus, ovary, testis, endometrium, hematopoietic cells, several cancer cell lines, all stages of the oocyte and, preimplantation embryos. After fertilization and during preimplantation development, *NLRP7* transcripts decrease to reach their lowest level at day 3 of embryonic development, which corresponds to the blastocyst stage, and then increase sharply from day 3 to day 5, which coincides with embryonic genome activation (EGA).

NLRP7 protein localizes mainly to the cortical region in all stages of human oocytes (36). In preimplantation embryos, NLRP7 localization becomes restricted to the outer region and is absent from the cell-to-cell contact region, similar to the localization of other proteins of the subcortical maternal complex (SCMC) (36).

1.2.2. *NLRP7* regulates interleukin-1 beta (IL-1β) secretion

The study of Kinoshita et al. was the first to establish a relationship between NLRP7 and IL-1β, and demonstrated that in stable transfections of THP-1 cells (of human monocytic origin) where

expressing an N-terminal 35-kDa NLPR7 fragment, which mimics some protein-truncating mutations observed in patients with RHMs, reduced IL-1β secretion which is an important cytokine in inflammatory responses (37). These findings are in line with those obtained by Khare et al. who demonstrated that *NLRP7* knockdown using small interfering RNA in macrophages significantly impairs IL-1β release upon stimulation with microbial acylated lipopeptides (38). However, NLRP7's anti-inflammatory role has been observed in non-immune cells. In two functional analyses by overexpression of NLRP7 in Human Embryonic Kidney 293 cells (HEK293) it was demonstrated that NLRP7 inhibited IL-1β release either by reducing the pro-IL-1β expression or by inhibiting Caspase 1 dependent IL-1β processing. Moreover, in another study on some HM patients carrying pathogenic *NLRP7* variants, in their peripheral blood mononuclear cells (PBMCs), despite having normal amounts of intracellular pro-IL-1β synthesis, the secretion of IL-1β was reduced compared to the cells from healthy individuals (35,39). In another study by Zhang et al, it was confirmed that homozygous or compound heterozygous NLRP7 mutations based on their existence in different domains of the protein, cause less IL1β secretion in the forms of either abnormal intracellular pro-IL-1β or mature IL-1β (40). Also, Messaed et al. pointed out that NLRP7 co-localizes with the Golgi and microtubule organizing center, and consequently associates with microtubules in monocytic cells. This observation suggests that *NLRP7* mutations may decrease cytokine secretion by affecting, directly or indirectly, the structure of cytoskeletal microtubules and impairing the trafficking of IL-1β (39).

NLRP7's roles during inflammation led the scientists to ask the question as to whether NLRP7's role in IL-1β production may be the cause of the early embryonic development arrest observed in HM patients. A growing body of evidence suggests that the interleukin-1 system components (IL-1 alpha, IL-1 beta, IL-1 receptors) have critical roles in ovulation, and oocyte maturation. It

has been demonstrated that intra-follicular injection of IL-1β increases the rate of ovulation but decreases the quality of the oocytes and consequently the rate of normal embryonic development (41). In contrast, this role for IL-1β in oocytes is in contradiction with data on cells from patients with NLRP7 mutations, which secrete lower amounts of IL-1β (39). Based on different studies so far, perhaps a combination of factors contribute to the formation of molar conceptions; some acting in the oocytes and others acting in hematopoietic inflammatory system affecting the differentiation/proliferation of embryonic/trophoblastic tissues and downregulating the maternal immune response, respectively (1). Because peripheral blood mononuclear cells from the patients fail to secrete normal amounts of IL1B, the patients fail to mount an appropriate inflammatory response to reject their arrested pregnancies and the delayed rejection of these pregnancies contributes to the molar phenotype (42). Additionally, *NLRP7* has been shown to promote cellular proliferation and myometrial invasion in testicular and endometrial cancer respectively (43).

1.2.3. *NLRP7* affects trophoblast differentiation and hCG level

Another interesting role for NLRP7 was demonstrated by Mahadevan *et al.* In this study, the authors showed that *NLRP7* knockdown accelerates trophoblast differentiation in human embryonic stem cells (hESC) by inducing the expression of GCM1 and INSL4, two trophoblast lineage markers (44). Furthermore, the amount of human chorionic gonadotropin (hCG) increased in knockdown hESC. These findings are striking since hydatidiform mole is characterized by hyperproliferation of the trophoblast and the production of high levels of hCG.

1.2.4 *KHDC3L*

In 2011, Parry and colleagues identified *KHDC3L* K homology (KH) domain containing 3 like; MIM 611687) as the second recessive gene responsible for familial biparental HMs (HYDM2; MIM: 614293) (45). To date, only seven mutations in *KHDC3L* have been reported in patients with RHM (Fig); therefore, this gene is considered as a minor gene for RHM accounting for 10-14% of patients who do not have mutations in *NLRP7*.

KHDC3L is a member of a 100 kb cluster on human chromosome 6 containing four related genes, *KHDC1* (KH domain containing 1 [MIM 611688]), *DPPA5* (developmental pluripotency associated 5 [MIM 611111]) and *OOEP* (oocyte expressed protein [MIM 611689]) with the following orientation from centromere to telomere (*KHDC1*, *DPPA5*, *KHDC3L* and *OOEP*) (Figure 2). Members of this gene family display mostly oocyte-or early embryo-specific expression pattern and the encoded proteins are characterized by an atypical K-homology (KH) domain that does not bind to RNA as opposed to canonical KH domain in corresponding proteins (46).

Phylogenetic analysis of the *KHDC1/DPPA5/KHDC3L/OOEP* orthologs showed this family does not exist in fish, chicken, or opossum; however, their existence in the eutherian mammalian genomes such as chimpanzee, macaque, dog, and rat suggests that the family has evolved rapidly and is in a particularly unstable genomic region (46).

KHDC3L transcripts have been identified to be maximal in GV oocytes, decrease through MII oocytes and are almost absent in pre-implantation embryos. KHDC3L protein is expressed in the cytoplasm of oocytes and pre-implantation embryos and localizes more specifically in the

cortical region than in the center of cytoplasm. Of note, this temporal pattern of expression of KHDC3L in oocytes is similar to that of NLRP7.

Filia, the mouse ortholog of *KHDC3L* is expressed in growing oocytes, encodes a protein that binds to MATER and forms the subcortical maternal complex (SCMC), which is essential for cleavage-stage embryogenesis. Filia null females have reduced fecundity and impaired preimplantation embryo development with a high incidence of aneuploidy. The aneuploidy was due to abnormal spindle assembly (1-polar, 3-polar, broad polar spindles), chromosome misalignment, and spindle assembly checkpoint inactivation which indicates Filia's role in maintaining chromosome stability and euploidy in early-cleavage mouse embryogenesis (47). In another study by downregulating KHDC3L expression via short interfering RNA (siRNA) injection into human immature oocytes, the KHDC3L's roles in spindle assembly and maturation/fertilization rate of human oocytes as well as the cleavage rate of the resulting zygotes were confirmed (48).

1.3. NLRP7/ KHDC3L and genomic imprinting

Normal mammalian development requires biparental genetic contributions because of the phenomenon of genomic imprinting in which the expression of some specific genes is dependent on whether the allele is transmitted from the sperm or from the oocyte. At these imprinted loci, different epigenetic modifications such as DNA methylation, histone modification or/and chromatin remodeling arise on the maternal and paternal alleles, resulting in differential gene expression.

The involvement of genomic imprinting in the pathology of hydatidiform moles was suspected soon after it was demonstrated that both sporadic androgenetic moles with two paternal genomes

(UPD for all 23 chromosomes) and diploid familial moles with one genome from each parent show the same phenotype. These findings led geneticists to hypothesize that the causative gene for recurrent moles would be responsible for setting and/or maintaining the maternal imprints in the oocytes (49). To date, seven studies have investigated DNA methylation in diploid biparental HM from patients with bi-allelic mutations in *NLRP7* or *KHDC3L* and revealed a general trend of lack of DNA maternal methylation marks on imprinted genes (45,49).

In a study by Judson et al, by methylation analysis of several DMRs and comparing them in BiHM, parthenogenetic and androgenetic DNA controls, this group showed the absence of methylation at a few maternally methylated DMRs, KCNQ1OT1, SNRPN, PEG1 and PEG3; and a normal methylation at the paternally methylated DMR, H19. They also tested whether the methylation abnormalities in BiHM is because of a defective maternal gametic imprinting or it is a post-implantation defect and found that there is true germline defect in oocyte because secondary methylations were normal or near normal (50). One year later in another study the same results were replicated. Moreover, in this study by analyzing SNPs, this group demonstrated that the abnormal patterns of methylation were on the maternal alleles in BiCHMs (51). In a study by Delgado et al. the author compared the methylation defects in BiHM due to *NLRP7* mutation with androgenetic HMs and found a total paternalization of all ubiquitous and placenta-specific DMRs in androgenetic moles versus the lack-of-methylation only at maternal DMRs in BiHMs. Interestingly, she also pointed out an "inter-RHM variation observation" in which RHMs from two sisters with the same missense mutations, as well as consecutive RHMs from one patient show allelic methylation differences. By comparing the epigenomes of these two types of moles and not seeing any methylation anomalies in patients' blood DNA with bi-

allelic mutation in *NLRP7*, she concluded that *NLRP7* is a maternal-effect gene and involved in imprint acquisition in the oocyte (49).

In the most recent study by Demond et al, by performing single-cell bisulfite sequencing (scBS-seq) on five oocytes from a patient with BiCHM due to a recessive *KHDC3L* mutation (c.1A>G), a genome-wide deficit of DNA methylation was observed in the oocyte. The novel finding in this study in comparison with the previous studies was that maternal gDMRs were affected similarly as other sequence features that become methylated de novo in oocytes, which indicates that there is no specificity of the primary defect towards imprinted loci. Moreover, the methylation analysis of a preimplantation embryo and molar tissue from the same patient showed that following fertilization, methylation defects at imprinted genes persist, while most non-imprinted regions of the genome recover near-normal methylation during post-implantation development. Finally, it was speculated that normal localization of DNA methylation factors in growing oocytes could depend upon an intact SCMC (52).

1.4. Subcortical Maternal complex

Although the zygote is formed by the fusion of the maternal and paternal pronuclei, early mammalian development is essentially under the "maternal command" from factors deposited in the ooplasm. This oocyte's maternal legacy reaches far beyond embryonic development and affects fetal and post-natal health through a yet poorly understood mechanism.

During oocyte growth and maturation, the proteins required for successful fertilization and early embryogenesis accumulate in the egg. After the egg is ovulated into the oviduct, it fuses with the sperm to establish the diploid embryo. At fertilization, both gametes are transcriptionally silent and embryonic gene expression is not detected until the 2-cell stage in mice and 4-cell stage in

humans (53,54). The subcortical maternal complex (SCMC) is a multi-protein complex expressed uniquely in oocytes and pre-implantation embryos that is essential for zygote progression beyond the first embryonic cell divisions. SCMC assembles during oocyte growth and persists at the periphery of the outermost cells of the cleavage-stage embryos (Figure 1.4 A) (55) and is absent from the inner cells and areas of cell-cell contact (56).

It has been known for a long time that all transcripts of the SCMC are encoded by maternal-effect genes (MEG) and required for the transition from maternal to zygotic program of development. Females carrying defects in any of the MEG are healthy, but at risk of reproductive failure, often due to early developmental arrest or imprinting disorders in their offspring. Furthermore, functional studies in mouse have also proven the fact that SCMC is encoded by MEGs, because females carrying mutations in single genes of the SCMC are sterile or sub fertile (57). The transcripts of SCMC components accumulate during oogenesis and their abundance is at its maximal level in fully grown oocytes. During meiotic maturation and ovulation, most of these transcripts are degraded and virtually none is detected by the 2-cell stage of embryogenesis. However, the encoded proteins, first observed in growing oocytes, persist during preimplantation embryogenesis up to the blastocyst stage of development (55,58).

Li et al in 2008 reported that the subcortical maternal complex (SCMC) comprises four maternally encoded proteins, (NLR family, Pyrin domain-containing 5 (NLRP5; also known as Maternal Antigen That Embryos Require, MATER, oocyte-expressed protein (OOEP; also known as Factor Located in Oocytes Permitting Embryonic Development, FLOPED), Transducin-Like Enhancer of Split 6 (TLE6), and KH (K homology) Domain Containing 3-Like (KHDC3L; also known as ES Cell-Associated Transcript I, ECAT1, C6orf221 or FILIA) (Figure 1.4) (55). Targeted mice lacking individual components of the SCMC arrest between zygotic and

cleavage stages which are recapitulated in Table 1.1. Nlrp5, is the first characterized maternal-effect genes in mice (59), which was later found to physically interact with KHDC3L (60), whilst OOEP physically binds to NLRP5 and to TLE6. The combined molecular weight of the four canonical SCMC proteins (NLRP5 ~125 kDa; OOEP ~18 kDa; KHDC3L ~38 kDa; TLE6 ~65 kDa) is less than that observed by fast protein liquid chromatography gel filtration (between 669 and 2000 kDa) suggesting that there are additional SCMC proteins and interacting partners to be identified (58). The subcortical localization of PADI6 (peptidyl arginine Deiminase 6), NLRP7 (NLR Family Pyrin Domain Containing 7) and NLRP2 (NLR Family Pyrin Domain Containing 2) is similar to canonical members and implicate them as other potential member of SCMC. *NLRP2* like and *NLRP5* and *NLRP7*, encode NLR family proteins, is highly expressed in oocytes and pre-implantation embryos and associated with various forms of reproductive wastage (58). While maternal mutations in *NLRP2* have been described in a mother with two offspring affected with Beckwith–Wiedemann syndrome (BWS) and one multi-locus imprinting disturbance (MLID) (61), embryos from Nlrp2 null female mice exhibit a range of methylation abnormalities at imprinted loci (62).

While null mutations in mouse *Mater* (59), *Floped* (55), *Tle6* (56) , or *Padi6* (63) lead to cleavage-stage embryonic arrest and female sterility, the absence of *NLRP2* (62), *KHDC3* (64) causes a subtle phenotype which includes delayed preimplantation development and decreased fecundity (56).

1.4.1. SCMC participates in F-actin meshwork regulation and mitochondria redistribution

In 2014, Yu et al. in a study on *Tle6* null mouse model observed that more than 90% of two-cell embryos had unequal-sized blastomeres and found that SCMC is required for the formation of the cytoplasmic filamentous actin F-actin meshwork which consequently controls the central

positioning of the spindle and ensures symmetric division of mouse zygotes. In this study, it was observed that in the SCMC null mutants, the activity of Cofilin (a key modulator of F-actin assembly) was disrupted, which consequently led to the off-center spindles(56).

Another interesting study which links the SCMC to the regulation of the F-actin meshwork was carried out by Fernandes and collaborators on *Nlrp5* null mice. They observed that ovulated oocytes lacking *Nlrp5* have altered localization of mitochondria, which were scattered throughout the cytoplasm, rather than concentrated in the subcortical layer. They showed that the SCMC may be involved in translocating mitochondria (and possibly other organelles) to the subcortical region of the oocyte probably through its interaction with the F-actin meshwork via Cofilin (65).

1.4.2. SCMC is involved in the regulation of translation

The SCMC's role in the regulation of translation was postulated after *PADI6* was identified as the fifth member of SCMC complex. PADI6 localizes to and is essential for the formation of the oocyte cytoplasmic lattices (CPLs), a fibrillar matrix composed of maternal contribution of ribosomes and mRNA, which is unique in mammalian oocytes and preimplantation embryos (63). It was shown that the abundance and localization of the ribosomal components is dramatically affected in two-cell embryos from *PADI6* null females and that de novo protein synthesis is also dysregulated in these embryos (66).

Another evidence which supports the SCMC's involvement in translation is the fact that OOEP/FLOPED and KHDC3/FILIA belong to a family of genes with several members, encoding similar proteins with an atypical KH domain RNA-binding that binds polynucleotides

and endogenous RNA in vitro. All these observations suggest the involvement of SCMC components in binding to mRNAs and their storage in the subcortical region (46).

1.4.3. SCMC in epigenetic reprogramming

SCMC's function in epigenetic reprogramming of zygotes was suggested when it was reported that mutations in several components of the SCMC such as *NLRP2* (61), *NLRP5* (67), and *PADI6* (68) in mothers lead to different types of imprinting disorders (Beckwith-Wiedemann and Angelman syndromes) and multi-locus imprinting disturbance (MLID) in offspring. Of note, mutations in *NLRP7* and *KHDC3L* are known as the causes of recurrent hydatidiform moles which is the severe form of imprinting disorders. While a broad loss of only maternally methylated imprints was observed in the molar tissues of *NLRP7* mutant patients, both maternally and paternally methylated imprinted DMRs are affected in MLID patients resulting from *NLRP5* mutations and in offspring from Nlrp2$^{-/-}$ female mice, suggesting roles of these two genes in post-zygotic maintenance (58).

1.5. Genetics of Androgenetic RHMs has started to become unraveled

In 2018, Nguyen et al, reported bi-allelic mutations in meiotic double-stranded break formation protein 1(*MEI1*) (MIM:608797), type 2 DNA topoisomerase 6 subunit B-like (*TOP6BL/C11orf80*) (MIM: 616109), and REC114 meiotic recombination (*REC114*) in five unrelated women with recurrent androgenetic complete hydatidiform moles and miscarriages as well as men affected by azoospermia in the case of Mei1 (3). Strikingly, all three genes play a key role in the double strand break (DSBs) formation during meiosis I.

1.5.1. *MEI1*

Mei1 (meiosis defective 1) is the first meiosis-specific mutation isolated by random mutagenesis in mice. Both *Mei1* mutant males and females are sterile, and spermatocytes display defects in meiotic chromosome synapsis, and arrest in meiotic prophase before entering pachynema. This group also found that the main reason that can explain the meiotic arrest in *Mei1* mutants is deficiency in DSB formation (69,70).

The meiotic phenotypes of *Mei1* mutants show sexual dimorphism in which meiosis is differentially impacted in oocytes and spermatocytes. In *Mei1* mutant ovaries unlike arrested spermatocytes, 6% of mutant oocytes proved capable of progressing to metaphase I and attempting the first meiotic division (70). Nguyen et al, reported that most of *Mei1* mutant oocytes have misaligned chromosomes on the spindles, 63% of them fail to extrude the first polar body (PB), 20% extruded morphologically abnormal first PB and some extruded all their chromosomes together with the spindle microtubules into the PB (empty oocytes). They also demonstrated that Mei1$^{-/-}$ oocytes are capable of fertilization and that 5% lead to androgenetic zygotes but all arrest at the 2- to 4-cell stage (19).

1.5.2. TOP6BL

TOPO6B-Like (TOPO6BL), which shares strong structural similarity to the Topo6B subunit of Topo6 DNA topoisomerase, interacts and forms a complex with SPO11, the ortholog of subunit A of Topo6 DNA topoisomerase (Topo6A), and is required for meiotic DSB formation. Topo6 topoisomerases belongs to the type IIB family of topoisomerases, which is essential for relaxation of negative and positive supercoiled DNA and DNA decatenation through cleavage and ligation cycles. Notably, all phenotypes observed in *Top6bl*$^{-/-}$ spermatocytes are identical to those reported in *Spo11*$^{-/-}$ mice, which is consistent with DSB formation defect and meiotic

arrest before pachytene stage. Some of these phenotypes have reduced γH2AFX levels, which indicates a deficiency in DSB formation and the presence of unsynapsed chromosome axes. However, unlike *Spo11*$^{-/-}$ oocytes, *Top6bl* mutant oocytes were largely depleted of primordial and primary follicles (71).

1.5.3. REC114

Meiotic Recombination Protein REC114 forms a complex with MEI4 then co-localize on the axes of *meiotic* chromosomes and is required for DSBs formation during meiotic recombination. Both spermatogenesis and oogenesis in *Rec114* mutant mice are defective due to deficiency in DSB formation. In humans, in addition to a homozygous splicing mutation in *REC114* in a patient with one miscarriage, two spontaneous CHMs, and one CHM after intrauterine sperm injection that was reported by Nguyen et al, (3), another group reported two novel homozygous mutations in two independent consanguineous families that are associated with female infertility (phenotypes including multiple pronuclei (MPN) formation during fertilization, early embryonic arrest and failed implantation of surviving embryos after IVF/ICSI) (72).

1.6. Ovum donation is a potential treatment for RHM patients

So far, only 16 live births have been reported in patients with mutations in *NLRP7*. Analyzing these mutations showed they have mild functional consequences on the protein (missense, splice or PTVs at the end of the protein), however, two of these patients experienced early neonatal death, and one exhibited intrauterine growth restriction (29,73–76). Because the primary defect in these patients is in their oocyte, ovum donation has been proposed to these patients as a potential reproductive option. To date 11 successful live births have been achieved by ovum donation (75,77–82).

1.7. Rationale and aims

Patients with recurrent hydatidiform moles experience a psychological toll on top of not experiencing to have their own biological children and this grief accelerates when this condition becomes repetitive. Identification of new genes responsible for RHM and dissecting the genetic heterogeneity of this condition are the main aims of this thesis:

Aim 1. Mutational screening for *NLRP7* and *KHDC3L* in patients with RHMs (Chapter 2 &3).

Aim 2. Performing Whole Exome Sequencing (WES) on negative patients for mutations in these two known genes and analyzing the data (Chapter 2 &3).

Aim 3. Searching for a second patient with mutations in any of the identified candidate genes from aim 2 by targeted sequencing and then exome sequencing in a cohort of 96 patients with milder defects (patients with one HM and at least 2 miscarriages or patients with at least 3 miscarriages)

Aim 4. Investigating the roles of the identified genes in RHM and female reproduction (Chapter 4)

About the genetics of recurrent hydatidiform moles, it was known that *NLRP7* and *KHDC3L* are responsible for 55% and 5% of patients with RHMs, respectively. In 2018, one year after I started my project, *MEI1*, *TOP6BL* and *REC114* were found in five unrelated patients as the causative genes responsible for recurrent androgenetic hydatidiform moles, miscarriages and infertility (3). At the same time, bi-allelic mutation in *PADI6* was found in a patient with one HM and 5 miscarriages by our lab (2). All these findings were an indication to keep looking for new candidates for RHM in the remaining ~40% of patients.

According to aim 1 and 2, I started my project by mutational screening of the patients for *NLRP7* and *KHDC3L* and submitted 35 negative DNAs for WES and analyzed the results. Findings of these 2 aims are distributed in chapter 2 in two manuscripts that were published in **a)** Clinical Genetics (PMID: 33583041) **b)** Journal of Assisted Reproductive and Genetics (PMID: 33751332).

After analyzing the exome data and finding candidate genes, targeted sequencing of candidate genes was done in September 2018 on a cohort of 96 patients with milder defects (patients with at least HM and recurrent miscarriages). During this process, we realized that this condition is highly heterogeneous since we were not able to identify mutations in the same gene in two patients. Therefore, we decided to submit all the samples directly for exome sequencing. Furthermore, we included patients with recurrent miscarriages in our exome sequencing based on 2 reasons: 1) The three miotic genes, *MEI1*, *TOP6BL* and *REC114* rather than associating with recurrent Hydatidiform moles they were found as the causatives for recurrent miscarriages and infertility. 2) *PADI6* also linked HM phenotype to miscarriages and female infertility in the patient we reported and showed us how related are these phenotypes regarding their genetics and the underlying mechanisms. While performing exome sequencing to find the second patient with mutation in any identified candidate gene, we found a homozygous protein truncating mutation in CCNB3 in a patient with 16 miscarriages which is reported in Journal of Medical Genetics (PMID: 34021051) in chapter 3.

In the 35 new exomes, I found a bi-allelic protein truncating mutation in *HFM1* and a conserved missense variant in *FOXL2*. Another lab member identified two protein-truncating mutations in *MAJIN* and *SYCP2* and compound heterozygous missense variants in *TOPBP1 in* patients with

recurrent hydatidiform moles. The results of this objective are presented in Chapter 3 and the manuscript is in preparation.

1.8. Figures and Tables

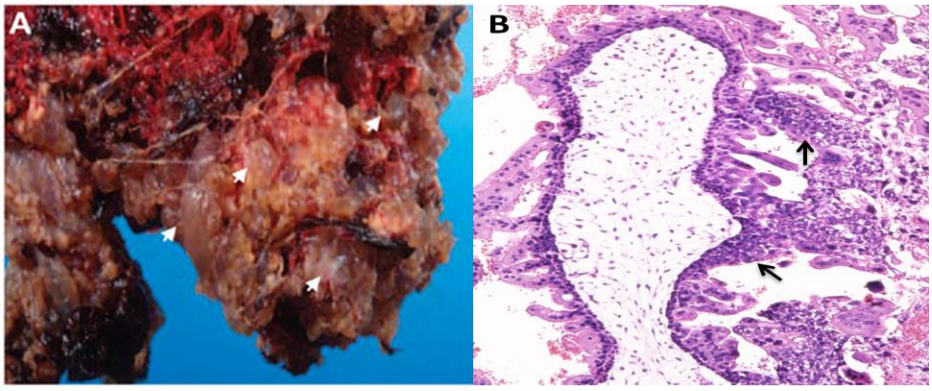

Figure 1.1. Gross-morphology of HM. (A) Gross-morphology of an HM directly after surgical evacuation. Note the presence of vesicles (some edematic chorionic villi (CV) are indicated by white arrows) that have accumulated fluid. (B) Histopathological cross-section of an HM showing circumferential trophoblastic proliferation (black arrows) around a chorionic villous (CV).

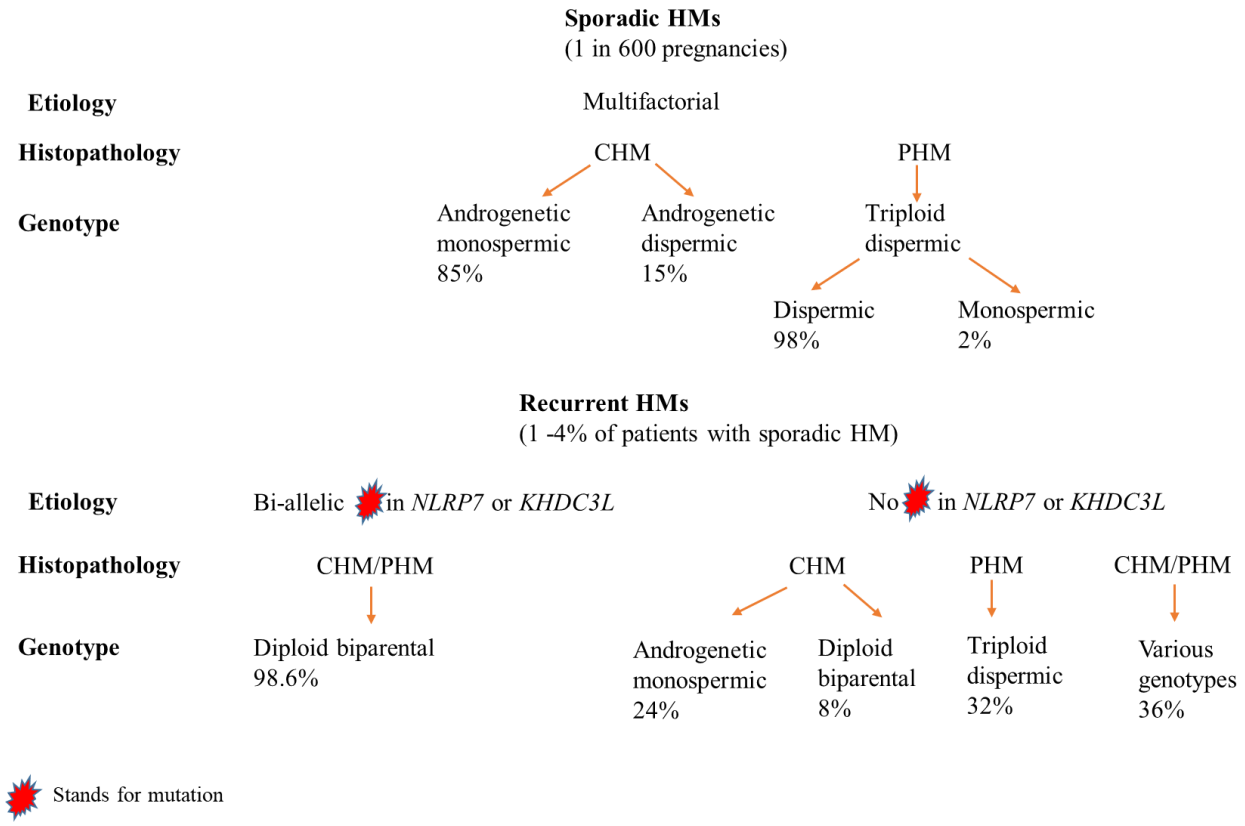

Figure 1.2. Classification of sporadic and recurrent HMs by etiology, histopathology, and genotype (18). CHM stands for complete hydatidiform ole; PHM, partial hydatidiform mole.

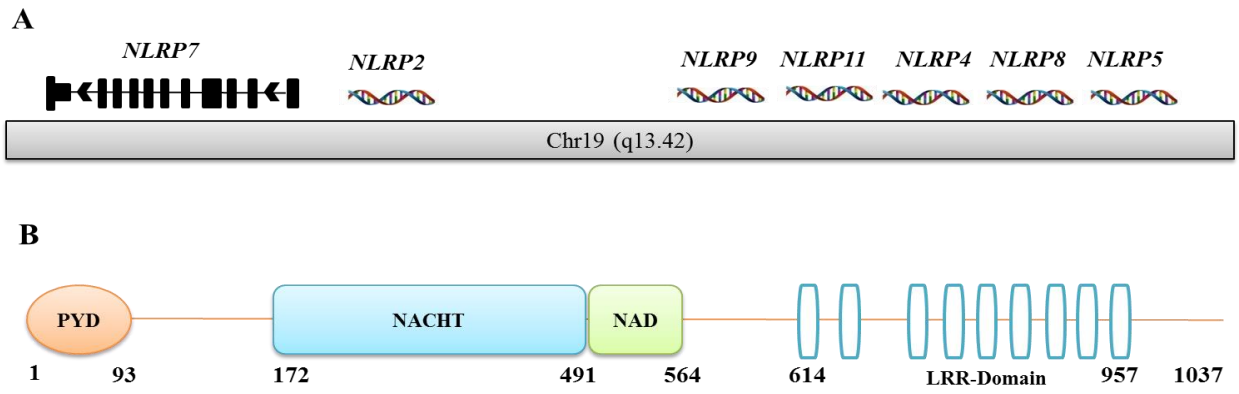

Figure 1.3. *NLRP7* genomic location and protein structure. (A) Schematic organization of NLRP7 along with other six NLRP genes in mammalian genome on human chromosome 19q13.42. (B) NLRP7 protein structure with different domains (PYD): pyrin domain; (NACHT): nucleoside-triphosphatase domain named after proteins NAIP, CIITA, HET-E and TP1; (NAD): NAD: NACHT-associated domain; (LRR): leucine-rich repeat).

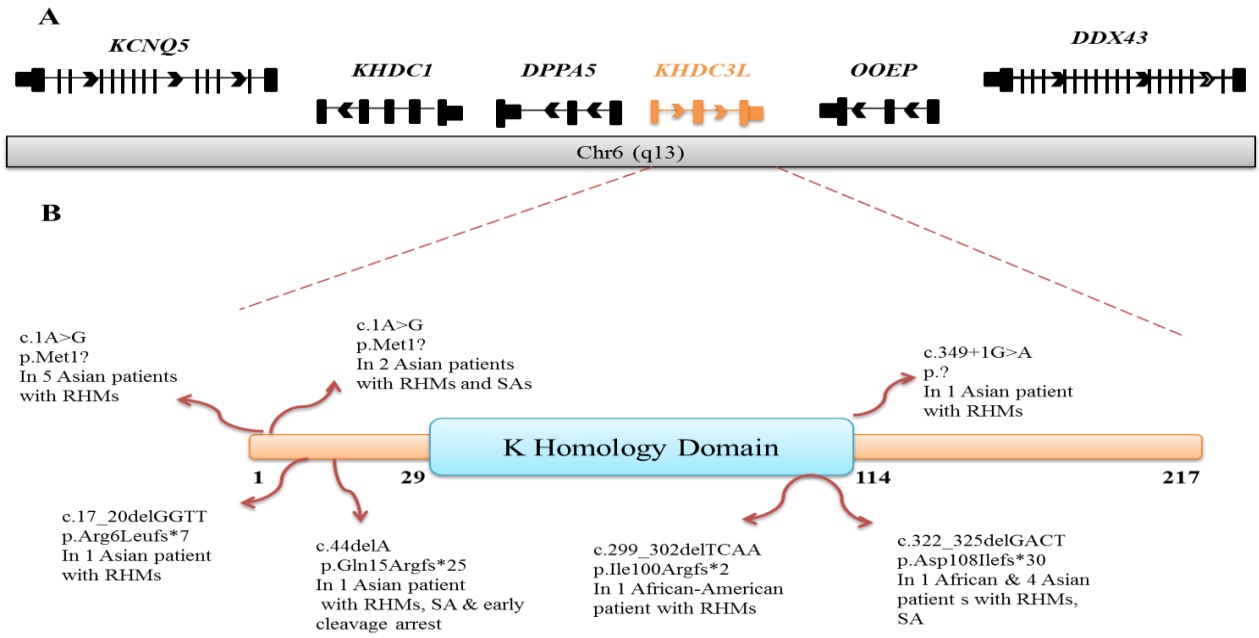

Figure 1.4. *KHDC3L* genomic location, protein structure and variants**.** (A) Schematic organization of *KHDC1/DPPA5/KHDC3L/OOEP* genes on human chromosome 6q13, in a locus

flanked by *KCNQ5* and *DDX43* genes. (B) KHDC3L protein structure consists of 217 amino acids and the locations of the 7 variants that have been reported in RHM patients.

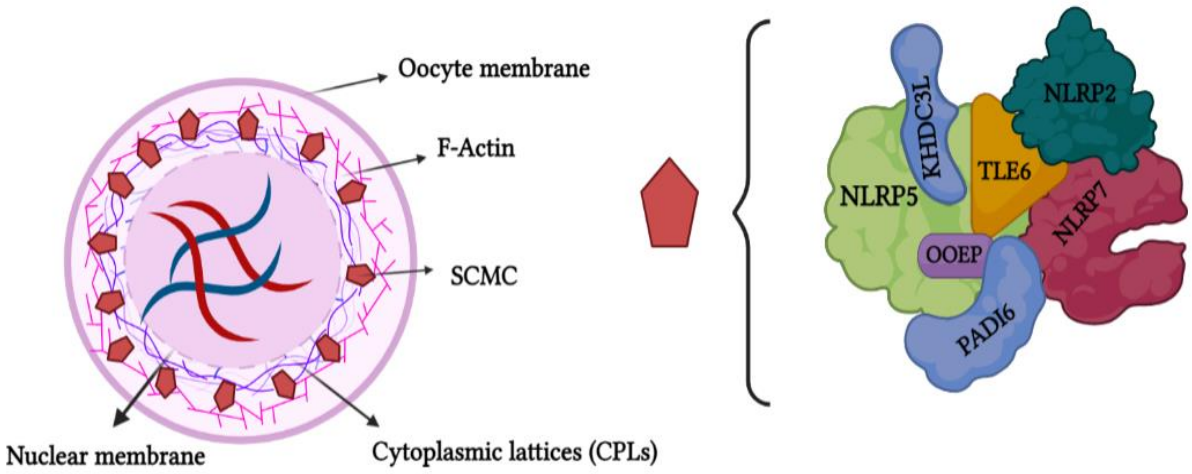

Figure 1.5. Subcortical maternal complex (SCMC)**.** (A) Shows the physical location of SCMC along with actin meshwork and cytoplasmic lattices (CPLs) under the cortex of oocyte membrane. (B) Demonstrates the SCMC members. NLRP5, OOEP, KHDC3L and TLE6 are the canonical members while PADI6, NLRP7, and NLRP2, are the potential members based on their functions and localization.

Tables

Human Gene	Specific function	Effects of genetic variants in human	Mouse Gene	Effects of null mutations in mouse
NLRP5 (MATER) 1200 aa	*CPL formation (Kim et al. 2010) *Mitochondrial localization and activity Kanzaki et al. (2020), Fernandes et al. (2012) *Organelle distribution Kim et al. (2014) *Oocyte DNA methylation Docherty et al. (2015)	*Unexplained infertility; *Embryonic arrest at the 2–7 cell stage *MLID Mu et al. (2019); Xu et al. (2020); Docherty et al. (2015)	Nlrp5 (Mater) 125 kDa, 1163 aa	*Cleavage-stage embryonic arrest and female sterility (Tong 2000)
KHDC3 (FILIA) 217 aa	*CPL formation, organelle distribution Qin et al. (2019) *Oocyte DNA methylation Demond et al. (2019)	*Recurrent hydatidiform moles Akoury et al. (2015), Parry et al. (2011); Demond et al. (2019)	Khdc3 (Filia) 38 kDa, 346 aa	*Delayed preimplantation development and decreased fecundity Li et al. (2008); Zheng &Dean et als. (2009)
OOEP (FLOPED) 149 aa	*CPL formation Tashiro et al. (2010)	*MLID Begemann et al. (2018)	Ooep (Floped) 18 kDa, 164 aa	*Cleavage-stage embryonic arrest and female sterility Li et al. (2008)
TLE6 449 aa	*CPL formation, formation of actin meshwork Alazami et al. (2015)	*Fertilization failure *Embryonic lethality at zygote, 2- or 4-cell stage Alazami et al. (2015); Lin et al. (2020)	Tle6 65 kDa, 581 aa	*Cleavage-stage embryonic arrest and female sterility Li et al. (2008)

Table 1. A recapitulation of the roles of four canonical members of subcortical maternal complex (*NLRP5*, *KHDC3*, *OOEP* and *TLE6*). The function of the genes in SCMC, the phenotypes of null human and mouse genes are summarized *(45,52,55,59,64,65,75,83–89)*.

PREFACE TO CHAPTER 2

In line with aims 1&2, the patients referred to our lab were screened in a 2- step process; first, by performing mutational screening for the known genes *NLRP7* and *KHDC3L* and second, by Whole Exome Sequencing (WES) of DNA of DNA of negative patients to search for additional novel genes. Using this approach, I performed mutation analysis on 35 patients and found 6 novel mutations in *NLRP7* that are listed in two manuscripts in this chapter.

During the mutation screening of some of the patients who were negative for mutations in *NLRP7* and *KHDC3L* and because they had a long region of homozygosity over all the NLRP7 SNP covered by Sanger sequencing, Droplet Digital ddPCR, long range PCR and sometimes CytoScan Xon microarray assay were performed to exclude the presence of large deletions and rearrangements in the major gene, NLRP7. This step is essential specially for *NLRP7* since it is highly rich in repetitive Alu elements and consequently is prone to large deletions and rearrangements (90).

Chapter 2 (first manuscript)

Novel pathogenic variants in *NLRP7, NLRP5* and *PADI6* in patients with recurrent hydatidiform moles and reproductive failure

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Abstract

Recurrent hydatidiform moles (RHMs) are human pregnancies with abnormal embryonic development and hyperproliferating trophoblast. Bi-allelic mutations in *NLRP7* and *KHDC3L*, members of the subcortical maternal complex (SCMC), explain the etiology of RHMs in only 60% of patients. Here we report the identification of seven functional variants in a recessive state in three SCMC members, five in *NLRP7*, one in *NLRP5* and one in *PADI6*. In *NLRP5*, we report the first patient with RHMs and bi-allelic mutations. In *PADI6*, the patient had four molar pregnancies, two of which had fetuses with various abnormalities including placental mesenchymal dysplasia and intra-uterine growth restriction, which are features of Beckwith-Wiedemann syndrome (BWS) and Silver Russell syndrome (SRS), respectively. Our findings corroborate recent studies and highlight the common oocyte origin of all these conditions and the continuous spectrum of abnormalities associated with deficiencies in the SCMC genes.

Key words: SCMC**,** Hydatidiform moles, Infertility, *NLRP7*, *KHDC3L*, *PADI6*, *NLRP5*, Imprinting disorders

Introduction

Hydatidiform mole (HM) is a human pregnancy with abnormal embryonic development, hydropic chorionic villi, and excessive trophoblastic proliferation. Based on microscopic morphological evaluation, HMs are divided into complete (CHM) and partial (PHM) depending mainly on the severity of trophoblastic proliferation and presence or absence of embryonic tissues. Recurrent HMs (RHMs) affects 1-9% of patients with a prior HM and refers to the occurrence of two or more HMs in the same patient. Recessive mutations in *NLRP7* and *KHDC3L*, are the major cause for RHMs and explain this etiology in 55% (29) and 5% (45) of patients, respectively. *NLRP7* and KHDC3L are members of the subcortical maternal complex (SCMC), a multi-protein complex uniquely expressed at the cortex of mammalian oocytes. The SCMC proteins orchestrate a variety of cellular processes that are essential for the activation of the zygotic genome (91).

To identify novel causative genes for RHMs, we screened patients with RHMs for known mutations in *NLRP7* and *KHDC3L*. Negative patients were then analysed by whole exome sequencing (WES). Here we report the identification of five novel recessive mutations in *NLRP7* and two novel recessive missense mutations in *NLRP5* and *PADI6*.

Methods

The study was approved by the McGill Institutional Review Board (IRB# A01-M07-03A). All methods were performed as previously described (92).

Results

NLRP7

NLRP7 screening in patients with RHMs by Sanger sequencing identified two missense variants, a duplication, a splice donor variant, and a large deletion in the 5'UTR in recessive state in five patients (Table 1; Supplementary figures 1-3).

NLRP5

Patient 1585 was negative for *NLRP7* and *KHDC3L* mutations. WES revealed a homozygous missense, c.1093G>A, p.(Asp365Asn), that affects a conserved amino acid in *NLRP5*. This variant is located in a 0.443 Mb run of homozygosity, which is consistent with the lack of known consanguinity between the patient's parents (Table 1, Figure 1A-C). Several attempts were made to obtain archived tissues from the products of conception (POCs) of this patient to re-evaluate the diagnosis, but without success.

PADI6

Patient 1678 was also negative for *NLRP7* and *KHDC3L* mutations, WES revealed a novel missense in *PADI6* (c.1796T>A, p.(Ile599Asn) (Table 1, Figure 1D-F), and two novel missense in *PADI1,* c.1928T>C, p.(Ile643Thr) and c.775G>T, p.(Ala259Ser). The three variants are located in a 13.1 Mb run of homozygosity (Supplementary figure 2). *PADI1* does not have

any functional role in reproduction and is therefore less likely to be responsible for her RHMs. *In silico* analysis of p.Ile599Asn in *PADI6* indicated that this variant is pathogenic by SIFT and conserved by GERP. Three other lines of evidence are in favor of its pathogenicity. First, this missense changes a non-polar amino acid, isoleucine, to a polar amino acid, asparagine. Second, the p.Ile599Asn is located in the protein arginine deiminase (PAD) domain, which is the most important domain of PADI proteins (68). Third, the missense at position 599 affects a conserved amino acid in primates and is just one amino acid after p.Asn598Ser, which we previously reported in a patient with bi-allelic *PADI6* missense variants and a history of one HM and five miscarriages (2). These observations suggest that the missense variant in *PADI6* most likely underlies the etiology of RHMs in patient 1678.

Patient 1678 had a total of seven pregnancy losses from spontaneous conceptions, four HMs including two with fetuses and three miscarriages. Only one POC (her 5^{th} pregnancy), which was referred to us as a PHM with a male fetus evacuated at 15-16 weeks, was available for analysis. Fetal autopsy had revealed dysmorphic facies (large forehead, hypertelorism, protuberant eyes, posteriorly rotated ears, long philtrum, midline cleft of soft palate with bifid uvula, receding chin, and short tongue), upper and lower limb abnormalities (thin with reduced muscle mass and prominent fetal pads in hands and feet), segmentation defect of the left lung, short pancreas, right kidney pyelectasia, X-ray fetogram (large vault with dolicocephaly and broad clavicles). Our morphological evaluation revealed two populations of chorionic villi (CV), molar and non-molar. Molar CV had circumferential trophoblastic proliferation and some were hydropic (Figure 2A- B) while non-molar CV did not have trophoblastic proliferation and were

not hydropic (Figure 2C). In addition, non-molar CV had fetal vessels with enucleated red-blood cells (Figure 2C). Fetal membranes and cord were present in this POC.

P57 is the protein encoded by the cyclin dependent kinase inhibitor 1C (*CDKN1C*), a negative regulator of cell proliferation that is expressed by the cells when they exit the cell cycle, stop proliferating, and acquire terminal differentiation. *CDKN1C* is an imprinted gene that is expressed only from the maternal allele in the cytotrophoblast and villous stroma cells of first trimester placenta. P57 is expressed in the cytotrophoblast and stroma cells of triploid PHM, which contains a maternal genome, but not in those of androgenetic CHM, which lacks a maternal genome. Consequently, since common HM are either triploid or androgenetic, p57 immunohistochemistry has been used as an ancillary marker to help classifying common HM into CHM and PHM. Evaluation of p57 expression in the POC of patient 1678 revealed that molar CV are negative in the cytotrophoblast and stroma cells (Figure 2D, E) and therefore fulfill the diagnosis of CHM. However, non-molar CV were positive in the cytotrophoblast but negative in the stroma (Figure 2F-H), which indicates that stroma cells of non-molar CV did not complete their differentiation and acquire p57 expression. This discordance between p57 staining and the differentiation of the cytotrophoblast and stroma cells is a feature of placental mesenchymal dysplasia (PMD), which is associated with BWS. We next attempted to determine the parental genomic contribution to this POC using various methods, but all our attempts failed due to non-optimal initial fixation of the tissues. In conclusion, based on these data, we confirm the diagnosis of HM and PMD in this POC.

The second POC of this patient was originally reported to us as a PHM with a fetus. This POC was diagnosed by ultrasonography at 15 weeks of gestation with placentomegaly and cystic molar changes. Amniocentesis revealed normal fetal karyotype. Follow-up ultrasound revealed

oligohydramnios, placental thickening, PMD, and severe intrauterine growth retardation (IUGR), which is a feature of SRS. Labor was induced at 32 weeks and the patient delivered a male baby who died 30 min later. By gross morphology, the newborn had ambiguous genitalia and hypospadias. Fetal autopsy also revealed several abnormalities including head to trunk size discrepancy and large forehead. Microscopic morphological evaluation of the placenta led to the diagnosis of a PHM with a fetus.

Discussion

Here we report seven novel functional bi-allelic variants, five in *NLRP7*, one in *NLRP5*, and one in *PADI6*. In *NLRP5*, we describe the first patient with a homozygous missense mutation, infertility, and three recurrent moles. The fact that the patient had three HM makes it unlikely that the diagnosis of the three HMs is wrong and is therefore in favor of the association of bi-allelic *NLRP5* mutations with RHMs that remains to be demonstrated in future studies.

In *PADI6*, we identified a novel missense variant in a patient with seven pregnancy losses, four HMs including two with fetuses that have features of BWS, and SRS. Bi-allelic mutations in *PADI6* were first identified in females with primary infertility and early embryonic arrest during cleavage stages after ART (93). Recently, we and others reported a patient with bi-allelic *PADI6* missense mutations and one HM and five miscarriages (2) and three women with bi-allelic *PADI6* mutations who had five children with BWS (68,94). In the latter study, the authors noted the variability in the pattern of aberrant imprinting in the offspring of these women in the number of impaired differentially methylated regions and the severity of their hypomethylation[5]. One study highlighted the atypical phenotype in one offspring who had some features of BWS and others of SRS (94), which is similar to our findings in the fetuses of patient

1678. Altogether, these data confirm the overlapping symptoms of BWS and SRS and support the emerging role for bi-allelic *PADI6* mutations in the causation of BWS, SRS, and imprinting disturbance.

NLRP7 is the first SCMC gene with causal role in female reproductive failure to be identified in humans. NLRP7 has been extensively studied with approximately 80 mutations observed in recessive state in a total of ~250 patients. *KHDC3L* is a minor gene for RHMs, only seven mutations in 16 patients have so far been reported. The earliest known defect in these patients is in their oocytes and is the impaired establishment of maternal methylation marks. This abnormality was originally believed to affect only imprinted genes; however, recent work has demonstrated that it affects similarly all *de novo* DNA methylation of the oocyte genome (95). The causal roles of the other SCMC genes, *TLE6*, *PADI6*, *NLRP5*, and *NLRP2*, in reproductive failure have recently been established. A recapitulation of all bi-allelic functional variants in these four genes with the reproductive histories of the patients are provided in Supplementary Table 1.These data show that the number of patients with mutations in all the SCMC genes, with the exception of *NLRP7*, are still small (five to 16) to draw meaningful correlations between the nature of the mutations and the reproductive outcomes. Despite this limitation and based on available cases, the main difference between the SCMC genes is that bi-allelic *NLRP7* and *KHDC3L* mutations have never been reported in probands presenting with primary infertility or recurrent miscarriages and no moles despite the screening of relatively large cohorts of patients with various forms of reproductive loss. However, 72% to 100% of patients with bi-allelic mutations in *NLRP5*, *NLRP2*, *PADI6*, and *TLE6*, had primary infertility. The main similarity is that all the SCMC genes, with the exception of *TLE6* (due to its association only with infertility with the latest observed developmental stage being biochemical pregnancy), have established

roles in DNA methylation in the oocytes. These data demonstrate clearly a continuous spectrum of conditions caused by mutations in the SCMC genes that originate in the oocyte and go beyond infertility and early pregnancy (e.g. HM or miscarriages), and include second and third trimester pregnancy losses as well as live birth with MLID.

BWS and SRS have always been believed to be pediatric conditions caused by mutations in the affected children. Linking BWS and SRS to recurrent moles strengthen the emerging thoughts that these two conditions, at least in some cases (and perhaps in more), stem from abnormal programing of the oocyte genome. This defect appears to impair first the differentiation of the placenta (e.g. PMD) and subsequently that of embryonic tissues. Our study calls for more attention to be placed on the reproductive histories of mothers of children with BWS and/or SRS. In cases of several pregnancy losses (e.g. infertility, miscarriages, moles), whole exome sequencing of the mother's genome is highly recommended.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interest.

Figures

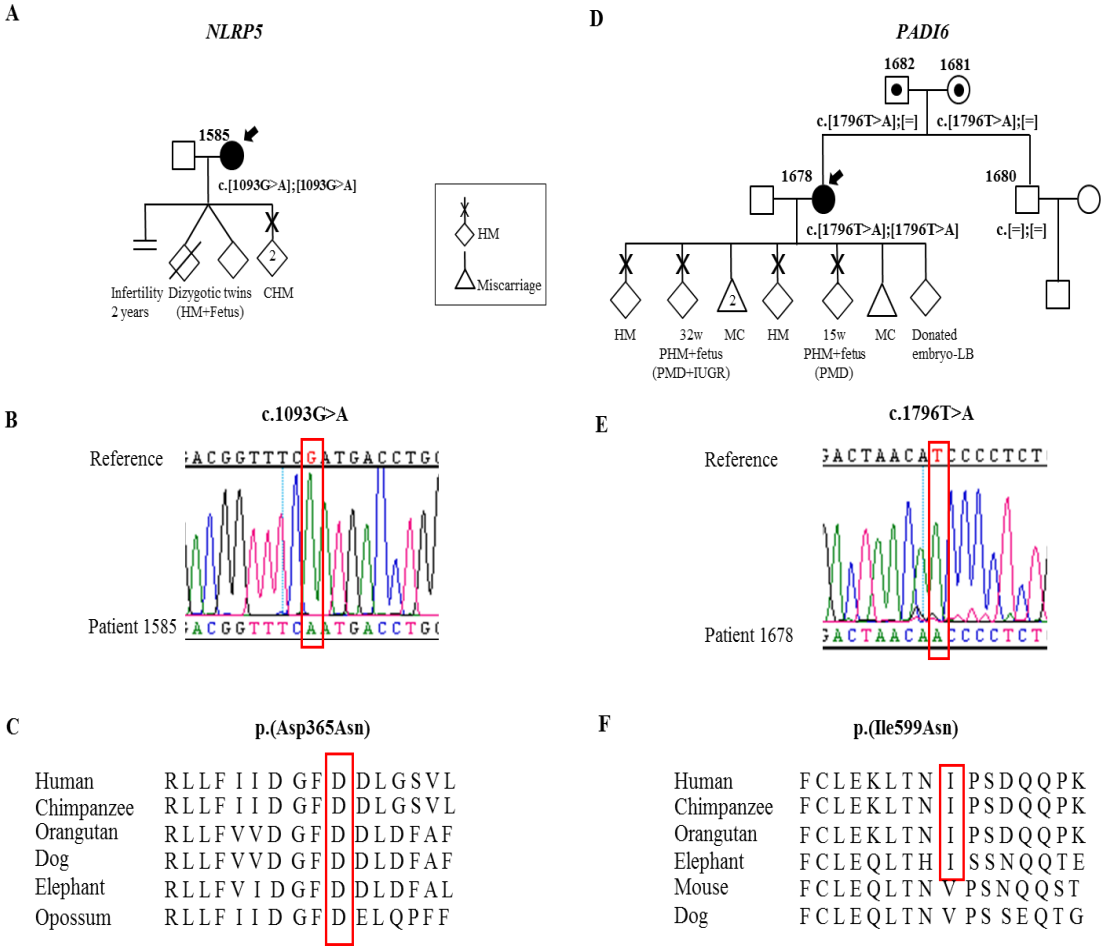

Figure 1. Chromatograms, segregation, and conservation of the missense variants in *NLRP5* and *PADI6*.

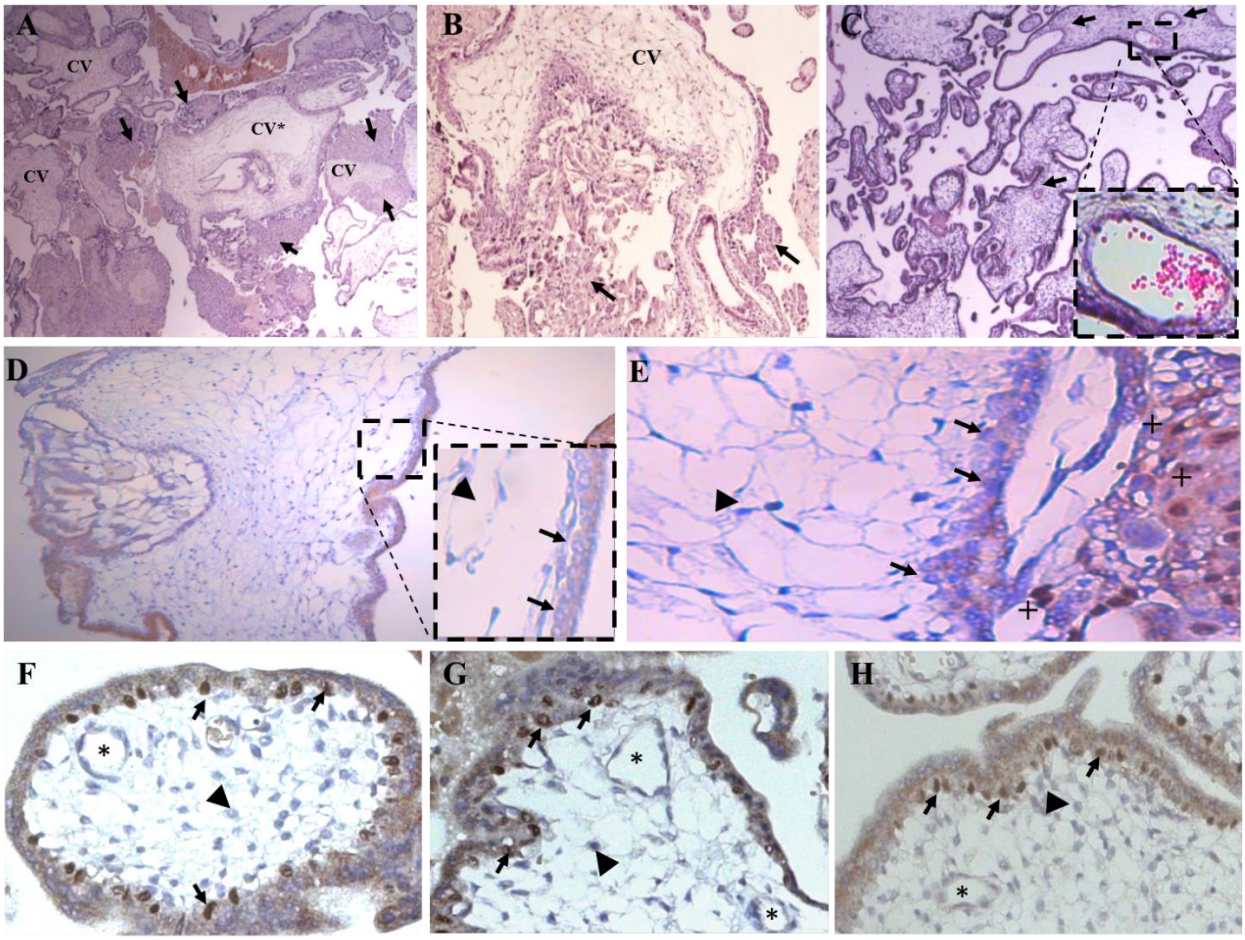

Figure 2. Morphology and p57 expression of one POC from the patient with PADI6 mutation**.** A-B) Microphotographs showing chorionic villi (CV) with circumferential trophoblastic proliferation (arrows) and one hydropic CV (asterisk). C) Presence of enucleated red-blood cells (inset) in non-molar CV. D and E show negative p57 staining in the nuclei of cytotrophoblast (arrow) and stroma cells (arrowhead) in molar CV. Nuclei of extravillous trophoblast cells are positive for p57 (+) and serve as an internal control. F-H show positive p57 cytotrophoblast (arrow) and negative stroma (arrowhead) cells in non-molar CV. Fetal vessels are indicated by asterisks.

Table 1 Variants in three genes coding for subcortical maternal complex proteins in patients with RHM											
Family ID	**Patient ID**	**Origin**	**Gene**	**Exon**	**Variants**		**gnomAD MAF**	**Polyphen score**	**CADD score**	**ACMG classification**	**Reproductive history**
					cDNA	Predicted protein					
MoIn759	1842	Indian	*NLRP7*	4	c.[1812_1837dup];[1812_1837dup]	p.[(His613Argfs*8)];[(His613Argfs*8)]	Absent	-	-	Likely Pathogenic	3 HM, ovum donation-on going normal pregnancy
MoEg695	1644	Egyptian	*NLRP7*	6	c.[2162G>A];[2162G>A]	p.[(Arg721Gln)];[(Arg721Gln)]	0.00001	0.87	12.35	Likely Pathogenic	SB, 2 CHM, CHM*, CHM-IM, LB, MC, HM
	1645 (sister 1)			6	c.[2162G>A];[2162G>A]	p.[(Arg721Gln)];[(Arg721Gln)]	0.00001	0.87	12.35		CHM, PHM, LB, PHM, MC, 2 LB (dizygotic twins with normal placentae)
	1646 (sister 2)			6	c.[2162G>A];[2162G>A]	p.[(Arg721Gln)];[(Arg721Gln)]	0.00001	0.87	12.35		Several MC
MoIr782	1856	Iranian	*NLRP7*	6	c.[2204A>C];[2204A>C]	p.[(His735Pro)];[(His735Pro)]	Absent	0.99	12	Uncertain significance	2 HM
MoCh566	1470	Chinese	*NLRP7*	5'UTR	c.[-40+3G>C]; [-40+3G>C]	p.(?);(?)	Absent			Uncertain significance	eTOP, CHM, CHM, several IVF failure
MoMx625	1543	Mexican	*NLRP7*	5'UTR/6	c.[-6831_-39-1586];[2248C>G]	p.[0];[(Leu750Val)]	Absent/ 0.0004	0.94	9	Pathogenic	2CHM, 2 PHM
MoIr673	1585	Iranian	*NLRP5*	7	c.[1093G>A];[1093G>A]	p.[(Asp365Asn)];[(Asp365Asn)]	Absent	1	24	Likely Benign	2 years of infertility, IUI-twin (HM+fetus), CHM, CHM
MoIn709	1678	Indian	*PADI6*	16	c.[1796T>A];[1796T>A]	p.[(Ile599Asn)];[(Ile599Asn)]	Absent	-	8.25	Uncertain significance	PHM, PHM with fetus (ultrasound at 32w showed IUGR, oligohydramnios, PMD), 2 MC, PHM* (Ultrasound at 15w showed cystic molar changes and head to trunck size discrepancy), MC, donor embryo-LB
W stands for weeks; HM, hydatidiform mole and is used when the classification was not available; PHM, partial HM; CHM, complete HM; MC, miscarriage; eTOP, elective termination of pregnancy; SB, stillbirth; END, early neonatal death; IUGR, intra-uterine growth restriction; PMD, placental mesenchymal dysplasia; LB, live birth; IUI, intra uterine insemination; IVF, in vitro fertilization; MAF, minor allele frequency. The absence of a number indicates one such reproductive outcome. The asterisk indicates the pregnancy that was available for us for evaluation. Variants nomenclature is given according to the following references, NM_001127255.1 for NLRP7, NM_153447 for NLRP5, and NM_207421 for PADI6.											

Table 1. Variants in 3 genes coding for SCMC in patients with RHM

Supplementary figure and table

Supplementary figure 1

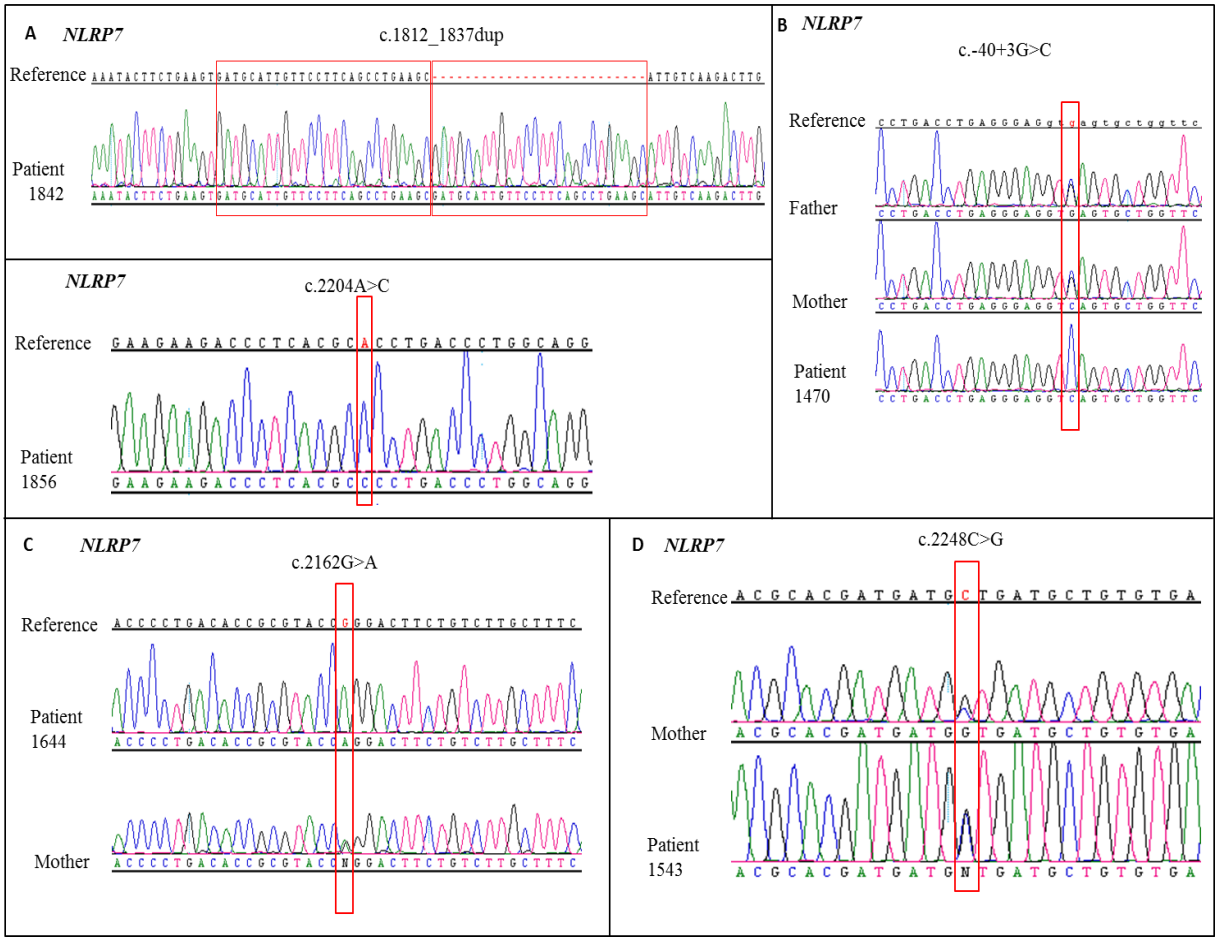

Figure S1. DNA sequencing chromatograms of *NLRP7* mutations**.** A) In the upper panel, in patient 1842, a homozygous mutation c.1812_1837dup was found. In the lower panel, in patient 1856, a homozygous mutation c.2204A > C was found. B) In patient 1470, a novel homozygous mutation, c.-40 + 3G > C, was found which was heterozygous in her parents. c.-40 + 3G > C affects the splice donor of NLRP7 exon 1, which is non-coding. In silico analysis of the effect of this variant, c.-40 + 3G > C, using NetUTR, indicated that it abolishes the splice donor site of exon 1 and probably leads to the inclusion of intron 1 in NLRP7 transcripts (which may impact their stability) or to the usage of some alternative splice site(s).C) c.2162G > A was found as a homozygous variant in patient 1644 and her 2 sisters, and heterozygous variant in their mother.

D) In patient 1543, a heterozgyous mutation, c.2248C > G, was found in exon 6 in a heterozgyous state. The second mutation in this patient was a large promoter deletion.

Supplementary figure 2

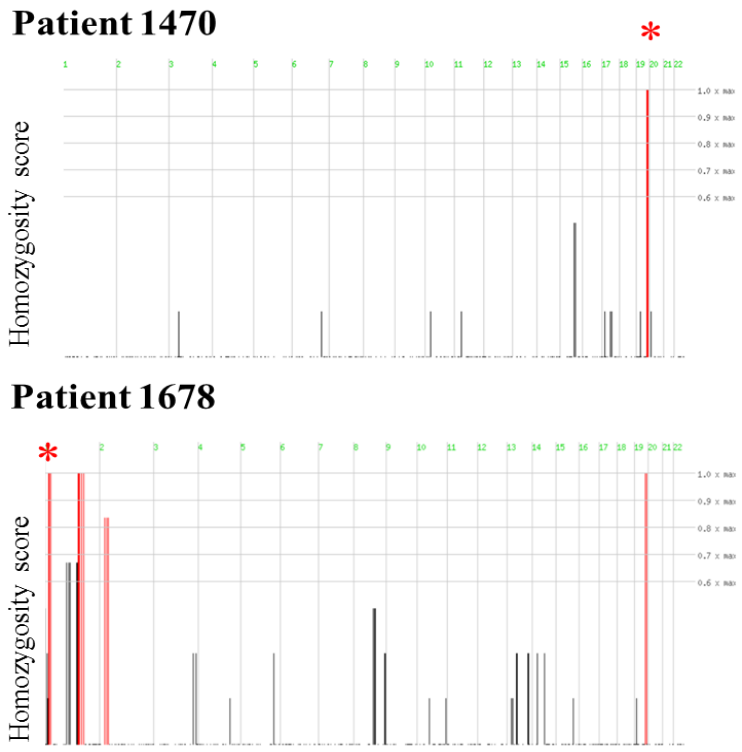

Figure S2. Homozygosity mapping in patients 1678 with *PADI6* mutation and 1470 with *NLRP7* mutation**.** The red lines represent homozygous regions that span 2 ≥ Mb (Seelow et al., 2009). The regions containing the causative genes are indicated by asterisks.

Supplementary figure 3

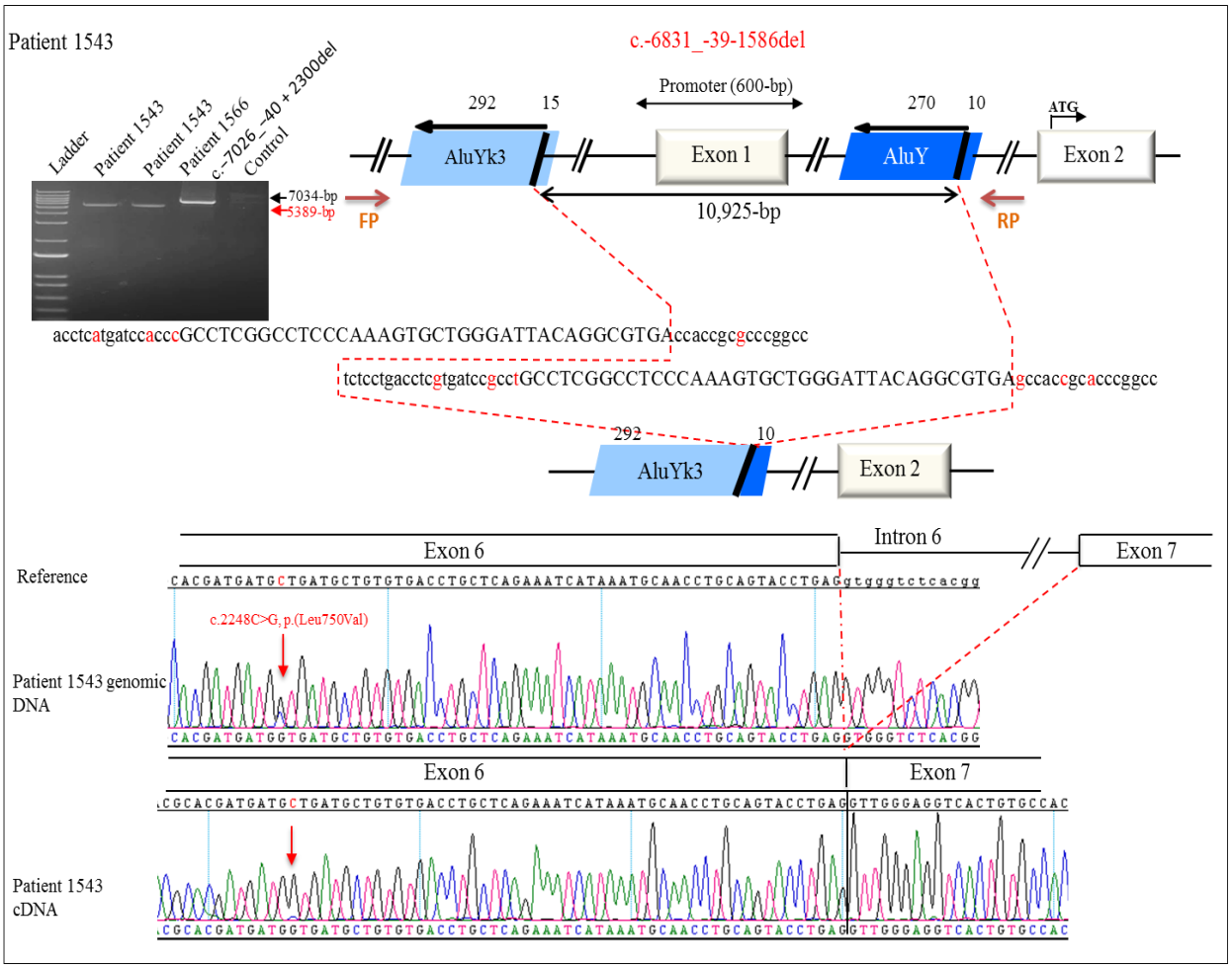

Figure S3. Characterization of the Alu mediated deletion in patient 1543 and schematic representation of the breakpoints**.** Top panel) ddPCR and chromosome walking by long-range PCR indicated a large Alu-mediated deletion in *NLRP7* 5'UTR which removes the transcription start site and predicted promoter region similar to a previously reported one in patient 1566 5. Long-range PCR amplification revealed a fragment of 5389-bp in patient 1543 and another of 7034-bp in patient 1566 (7034-bp) that were not amplified in a control subject. Sequencing the 5389-bp fragment showed that the deletion is mediated by recombination between two Alu Y elements and a microhomology of 37-bp, shown in capital letters. Lower panel) Sanger sequencing after RT-PCR of the region containing the other allele, c.2248C > G, p. Leu750Val,

in a heterozygous state showed its presence in a homozygous state indicating the absence of transcripts from the allele carrying the promoter deletion.

Supplementary table 1: Summary of all the variants* and their corresponding reproductive history in maternal effect genes				
Gene	**Protein change**	**Reproductive history**	**Reference**	**Summary of analyzed patients and results**
TLE6	p.[Arg409Gln];[Arg409Gln]	Primary infertility, multiple failed IVF/ICSI cycles	Lin et al, 2020	3/403 WES in patients with PI and early cleavage arrest
	p.[Glu541Lys];[Glu541Lys]	Primary infertility, multiple failed IVF/ICSI cycles		
	p.[Asp130Asn];[Val503Ile]	Primary infertility, multiple failed IVF/ICSI cycles		
	p.[Ser510Tyr];[Ser510Tyr]-Sister 1	Primary infertility, 4 failed ICSI cycles	Alazami et al, 2015	3/3 WES in patients with PI and early cleavage arrest
	p.[Ser510Tyr];[Ser510Tyr]-Sister 2	Primary infertility, 4 failed ICSI cycles		
	p.[Ser510Tyr];[Ser510Tyr]	Primary infertility, 2 failed ICSI cycles		
	p.[Ala378Glufs*75];[Ala378Glufs*75]	3 failed IVF cycles	Wang et al, 2018	1/4 WES & 0/80 TS in patients with PI and early cleavage arrest
	p.[Lys146Glufs*51];[Lys146Glufs*51]	2 failed IVF cycles, 4 biochemical pregnancies	Maddirevula et al., 2020	1/75 WES in patients with PI and early cleavage arrest
	p.[Gln74His];[Gln74His]	Primary infertility, 3 failed IVF cycles	Zheng et al, 2020	5/50 WES in patients with PI and early cleavage arrest
	p.[Gln74His];[p.Ala240Gly]	Primary infertility, 1 failed IVF cycle		
	p.[p.Asp522His];[p.Asp522His]	Primary infertility, 3 failed ICSI cycles		
	p.[Arg338His];[p.Trp446*]	Primary infertility, 2 failed IVF/ICSI cycles		
	p.[Thr298Arg];[Thr298Arg]	Primary infertility, 2failed IVF cycles		
PADI6	p.[Leu375Phefs*13];[Leu375Phefs*13]	Primary infertility, 2 failed ICSI cycles	Wang et al, 2018	2/4 WES & 0/80 TS in patients with PI and early cleavage arrest
	p.[Pro289Leu];[Pro632Leu]	Primary infertility, one failed IVF cycle		
	p.[Arg457*];[Arg457*]	Primary infertility, 2 failed ICSI cycles	Maddirevula et al, 2017	1/75 WES in patients with PI and early cleavage arrest
	p.[Gln381*];[Gln381*]-proband	Primary infertility, 6 failed IVF/ICSI cycles	Xu et al, 2016	3/5 WES & 2/36 TS in patients with PI and early cleavage arrest
	p.[Gln381*];[Gln381*]-sister 1	Primary infertility		
	p.[Gln381*];[Gln381*]-sister 2	Primary infertility		
	p.[Glu670Glyfs*48];[His211Gln]	Primary infertility, 3 faield ICSI cycles		
	p.[Gly540Arg];[Gln324*]	Primary infertility, 5 failed IVF/ICSI cycles		
	p.[Thr373Pro];[Arg570Cys]	Primary infertility, 2–3 failed IVF/ICSI cycles	Zheng et al, 2020	2/2 WES in patients with PI and early cleavage arrest
	p.[Ser508Glnfs*5];[Ser508Glnfs*5]	Primary infertility, 2 failed ICSI cycles		
	p.[Arg682Gln];[Asn598Ser]	5 MC, HM	Qian et al, 2018	1/77 WES in patients with RHMs, 0/13 WES & 0/53 TS in patients with ≥ 2MC
	p.[Ile599Asn];[Ile599Asn]	PHM, early neonatal death (IUGR, oligohydramnios, PMD), 2 MC, HM, IUI-HM, MC, donor embryo-LB	Current study	1/77 WES in patients with RHMs, 0/13 WES & 0/53 TS in patients with ≥ 2MC
	p.[Pro694Ser];[Met477Val]	BWS-girl, MC (20w), LB	Cubellis et al, 2022	2/6 WES in patients with MC/LB/MLID
	p.[Trp356*];[Pro632Ala]	2 BWS-girls (polyhydramnios, placental hyperplasia, macroglossia, umbilical hernia, lateralized overgrowth)		
	p.[Thr372Ala];[p.(Trp690*]	2 MLID-girls, 3 MC	Eggermann et al, 2020	1/1 WES in patients with MC/MLID
	p.[Ser278Profs*59];[Ser278Profs*59]	Primary infertility, 1 failed ICSI cycle	Zheng et al, 2020	1/50 WES in patients with PI and early cleavage arrest
NLRP5	p.[Trp759Aspfs*4];[Trp759Aspfs*4]	Primary infertility, 2 failed ICSI cycles	Maddirevula et al, 2020	1/75 WES in patients with PI and early cleavage arrest
	p.[Gln98*];[Thr694Ile]	Primary infertility, 3 failed IVF cycles	Mu et al, 2019	1/5 WES & 4/496 SS in in patients with PI and early cleavage arrest
	p.[Gly289Glu];[Thr1107Ile]	Primary infertility, 3 failed ICSI cycles		
	p.[Arg533Pro];[Leu640Arg]	Primary infertility, 2 failed IVF cycles	Li et al, 2020	1/1 WES in patients with PI and early cleavage arrest
	p.[Asp365Asn];[Asp365Asn]	Primary infertility, IUI-twin (HM+fetus), CHM, CHM	Current study	
	p.[Gln785*];[Leu947Pro]	4 MC, BWS-MLID boy, A boy with non-specific developmental and marked behavioural problems, 2 LB	Docherty et al, 2015	1/39 WES & 0/33 SS in patients with RPL/MLID
	p.[Cys774Arg];[Gly555Val]	6 MC and one HM with three unrelated partners, SRS-MLID girl, BWS-MLID boy		
	p.[Arg353*];[Arg533Cys]	3 MC, BWS, MLID boy, MC	Sparago et al, 2019	1/3 WES in patients with RPL/MLID
	p.[Arg635Cys];[Arg635Cys]	Primary infertility, 2 failed IVF/ICSI cycles	Zheng et al, 2020	3/50 WES in patients with PI and early cleavage arrest
	p.[Ser893Thr];[p.Leu1116Trp]	Primary infertility, 2 failed IVF/ICSI cycles		
	p.[Arg143Pro];[p.Arg462Cys]	Primary infertility, 2 failed IVF/ICSI cycles		
NLRP2	p.[Arg752*];[Phe258Ser]	Primary infertility, 2 failed IVF cycles	Mu et al, 2019	1/5 WES & 4/496 SS in in patients with PI and early cleavage arrest
	p.[Thr221Met*];[Glu616Val]	Primary infertility, 3 failed IVF/ICSI cycles, ICSI-LB		
	p.[Thr221Met*];[Arg490Cys]	Primary infertility, MC, 3 failed IVF/ICSI cycles, 2 ICSI-LB		
	p.[Trp175Cys];[Glu848Asp]	Primary infertility, 6 failed ICSI cycles		
	p.[Arg493Serfs*32];[Arg493Serfs*32]	2 BWS boys with polyhydramnios, A probable HM & MLID, LB	Meyer et al, 2009	1/14 SS in patients with BWS
	p.[Arg115*];[Arg115*]	Primary infertility, 2 failed IVF/ICSI cycles	Zheng et al, 2020	1/50 WES in patients with PI and early cleavage arrest

* In this table, we have only considered functional variants identified in a recessive state to be more accurate in our comparisons.Abbreviations: PI; Primary infertility; In vitro fertilization (IVF); Intracytoplasmic sperm injection (ICSI); Miscarriage (MC); Hydatidiform mole (HM); Partial hydatidiform mole (PHM); Complete hydatidiform mole (CHM); Intrauterine growth retardation (IUGR); RPL: Recurrent pregnancy losses; Intrauterine insemination (IUI); Live birth (LB); Beckwith-Wiedemann syndrome (BWS); Silver-Russell Syndrome (SRS); Multi-locus imprinting disturbances (MLID); Human chorionic gonadotropin (hHCG);Whole exome sequencing (WES); Targeted sequencing (TS); Sanger sequencing (SS). In the last column, the number before the slash (/) indicates the number of positive patients and the number after the shash indicates the number of screened patients. For example, "3/403" indicates that 3 patients were positive for PADI6 among 403 patients with PI and early cleavage arrest screened by whole exome sequencing.

Table S1. Summary of all the variants* and their corresponding reproductive history in maternal-effect genes

Supplementary Methods

Patients

Patients with at least two hydatidiform moles (all forms combined) were referred to our laboratory from various collaborators. All patients provided written consent to participate in our study, gave blood samples for mutation analysis, and agreed for us to retrieve their products of conception from various histopathology laboratories for research purposes.

Whole-exome-sequencing and Sanger sequencing

Exome sequencing was performed at the McGill University and Genome Quebec Innovation Center (Montreal, Canada). Either Roche Nimbelgen SeqCap EZ Human Exomes or MedExomes capture kits were used for Exome enrichment. Exome library was then sequenced with 100-bp paired-end reads on the Illumina Novaseq6000 sequencer and the reads were aligned to the human reference genome (hg19). Mutations were filtered (below table) using the following criteria: minor allele frequencies (< 0.005 in the Genome AD database), exonic non-synonymous or splice site variants, or coding insertions or deletions (indels), or expressed specifically or at high levels in oocytes.

PADI1 and *PADI6* variants in patient 1678 and *NLRP5* variant in patient 1585 were validated by Sanger sequencing in the affected individuals and their segregation checked in available family members.

Position	Variation	Ref	Alt	#alt b	#reads	Homozyg	In ROH	Protein Cl	Gene	Prev seen it	ExAC AF	gnomAD G	SIFT score	Polyphen2	CADD score
chr1:17725288	NSNV	T	A	49	49	hom	1	p.I599N	PADI6	1678	0	0	0	0	8.259
chr1:17555242	NSNV	G	T	46	46	hom	1	p.A259S	PADI1	1678	0.0001	0	0.81	0.254	14.41
chr1:17570674	NSNV	T	C	145	145	hom	1	p.I643T	PADI1	1678	0	0	1	0.555	26.1
chr19:56538692	NSNV	G	A	39	39	hom	1	p.D365N	NLRP5	1585	0	0	1	0.997	24

*NSNV stands for non-synonymous variant

Droplet Digital PCR (ddPCR) and Reverse transcription PCR (RT-PCR)

Reactions containing EvaGreen Master Mix (Bio-Rad, Hercules, CA, USA), primers, and template DNA were converted to droplets with the QX200 droplet generator (Bio-Rad). These samples were then transferred to a 96-well plate and heat-sealed and PCR performed as follows: initial denaturation at 95°C (10 min), amplification (40 cycles) at 95°C for 30 seconds and 60°C for 1 min, and enzyme deactivation at 98°C (10 min). The droplets were analyzed in the QX200 droplet reader (Bio-Rad) and analyzed with Quantasoft software 1.7 (Bio-Rad).

RT-PCR was performed on RNA extracted from Epstein-Barr virus (EBV) transformed cells of patient 1543 using Trizol (Invitrogen, Carlsbad, CA, USA) and verified by electrophoresis. cDNA was synthesized using a reverse transcription kit (Life Technologies, Carlsbad, CA, USA) and PCR performed using primers located in exons 5 and 7 of NLRP7. The amplified fragment was then sequenced by Sanger sequencing.

Chapter 2 (second manuscript)

The genetics of recurrent hydatidiform moles in Mexico: further evidence of a strong founder effect for one mutation in *NLRP7* and its widespread

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Abstract

Purpose

To investigate the frequency of a founder mutation in *NLRP7*, L750V, in independent cohorts of Mexican patients with recurrent hydatidiform moles (RHMs).

Methods

Mutation analysis was performed by Sanger sequencing on DNA from 44 unrelated Mexican patients with RHMs and seven molar tissues from seven additional unrelated patients.

Results

L750V was present in homozygous or heterozygous state in 37 (86%) patients and was transmitted on the same haplotype to patients from different states of Mexico. We also identified a second founder mutation, c.2810+2T>G in eight (18.1%) patients, and a novel premature stop-codon mutation W653*.

Conclusion

Our data confirm the strong founder effect for L750V, which appears to be the most common mutation in *NLRP7*. We also report on six healthy live births to five patients with bi-allelic *NLRP7* mutations, two from spontaneous conceptions and four from donated ovum and discuss our recommendations for DNA testing and genetic counseling.

Supplementary Information

The online version contains supplementary material available at 10.1007/s10815-021-02132-1.

Keywords: Recurrent hydatidiform moles, Founder mutation

Introduction

Hydatidiform mole (HM) is an aberrant human pregnancy characterized by abnormal embryonic development and excessive proliferation of the trophoblast. Common HM is sporadic and affects 1 in every 600 pregnancies (96). At the histopathological level, HM is classified as complete or partial. Complete hydatidiform moles (CHMs) are characterized by the absence of embryo and excessive proliferation of the trophoblast. Partial hydatidiform moles (PHMs) have moderate focal trophoblastic proliferation and may contain embryonic tissues. CHMs are androgenetic while PHMs are triploid dispermic (97). Recurrent hydatidiform moles (RHMs) are defined by the occurrence of at least two molar pregnancies in the same patient and affect approximately 1-9.4% of women with a prior HM, depending on studies and populations (98–102). Based on morphological analysis, RHMs may be classified as CHM or PHM.

Bi-allelic *NLRP7* mutations are the major cause for RHMs (OMIM 231090) (29) and explain the genetic etiology of 55% of patients (92). A second gene responsible for RHMs, *KHDC3L*, was identified in 2011 (45) and its bi-allelic mutations explain the etiology of 5% of patients with RHMs (hydatidiform mole, recurrent type 2 (OMIM 611687) (92,103). Molar tissues from patients with mutations in *NLRP7* or *KHDC3L* are diploid biparental. Both genes are components of the subcortical maternal complex, which is essential for epigenetic reprogramming of the oocyte genome and the activation of the embryonic genome (36,91,95). Recently, bi-allelic mutations in three other genes, *MEI1*, *TOP6BL* (C11orf80), and *REC114*,

with roles in meiotic double-strand break formation have been identified in patients with recurrent androgenetic complete hydatidiform moles, miscarriages, and infertility (3).

In 2013, our group analyzed *NLRP7* mutations in 20 Mexican patients with RHMs and found that 17 of them have bi-allelic mutations in *NLRP7* (78) and all the 17 patients had at least one copy of a previously reported mutation, c.2248C>G, p.Leu750Val (L750V) in two Mexican patients (104). Furthermore, of the 17 patients, 12 were homozygous for L750V. These 12 patients were born in different parts of Mexico and all denied consanguinity between their parents. In addition, the L750V was found in a heterozygous state in 5% of control subjects from the general Mexican population (78). These data suggested a strong founder effect for L750V in the Mexican population.

Founder mutations in *NLRP7* have been reported in other populations, including the Indian [c.2078G>C, p.(Arg693Pro) and c.2738A>G, p.(Asn913Ser)] and Egyptian [c.-39-387_2129+265dup, p.(Glu710Aspfs*7)] populations (73,104,105). However, the founder effect in the Mexican population appeared stronger because the same mutation was found in all the 17 patients with bi-allelic mutations we reported in Estrada et al. (78). We therefore set up to analyze another independent cohort of 44 unrelated Mexican patients with RHMs, and seven molar conceptions from unrelated patients with RHMs. Thirty-one of these patients and the seven moles were recruited or retrieved from the Instituto Nacional de Perinatologia in Mexico City. We also reviewed the mutation analysis results of another cohort of 13 unrelated patients with RHMs of Mexican origin who were referred either from the USA or Mexico to the Research Institute of the McGill University Health Centre (RI-MUHC) for mutation analysis. Our data

confirm our previous findings and highlight the strong founder effect for L750V in Mexico and its inheritance on the same haplotype to patients from various states. Our study also revealed a second founder mutation, c.2810+2T>G and a novel protein-truncating mutation in the Mexican population.

Material and methods

Patients with RHMs

The study was approved by the review boards of the Instituto Nacional de Perinatologia (INPer), study number: 212250-3220-11108-01-14 and McGill University (study number: A01-M07-03A). Patients with at least two HMs were referred from different hospitals in Mexico. A complete clinical evaluation including family and reproductive histories of the patients and their first-degree relatives was taken for all patients. When possible, sisters with RHMs and parents were invited to participate in the study. Written informed consents were obtained from all participants prior to obtaining venous blood samples. A total of 44 unrelated patients were included in this study, 31 were referred to INPer, and 13 were referred to the RI-MUHC. Archived formalin-fixed paraffin embedded (FFPE) molar tissues were retrieved from seven patients with RHMs from the INPer by screening the pathology department record for patients with RHMs.

DNA extraction and mutation analysis

Genomic DNA was isolated from the patient venous peripheral blood. Sequence analysis was performed at the INPer (Mexico) first for exon 6 of *NLRP7* to investigate the presence of the founder mutation L750V. Patients without bi-allelic mutations were screened for mutations in the other exons, 1 to 5 and 7 to 11, at the RI-MUHC (Montreal, Canada). Primer sequences and

polymerase chain reaction (PCR) conditions were as previously described (104,106) (Supplementary Table 1). PCR products were purified and directly sequenced in forward and reverse orientations using terminator dye in an ABI Prism 3130 (Applied Biosystems). All identified mutations were compared with the reference sequence NM_001127255.1 (http://fmf.igh.cnrs.fr/ISSAID/infevers/) and annotated according to the Human Genome Variation Society (HGVS) (http://varnomen.hgvs.org/). Sequence variant nomenclature is given according to the following references: NM_001127255.1 (cDNA), NG_008056.1 (genomic DNA), and NP_001120727.1 (protein). Patients who were negative for mutations in *NLRP7* were analyzed for mutations in *KHDC3L* as previously described (74).

Parental contribution to the molar tissues

Sections of FFPE molar tissues were stained with hematoxylin and eosin. Chorionic villi were separated from maternal tissues under a stereomicroscope and used to extract DNA as previously described (18,92). Multiplex microsatellite DNA genotyping was performed using the Powerplex 16 HS System (Promega Corporation, Fitchburg, WI, USA), and analyzed as previously described (18,92).

Results

During the study period, a total of 31 unrelated patients with RHMs were recruited and analyzed for mutations in NLRP7 (Table1). Of these 31 patients, seven had a family history of RHMs and nine (29%) patients had gestational trophoblastic disease after one of their molar pregnancies. Mutation analysis on these 31 patients revealed bi-allelic *NLRP7* mutations in 26 (83.8%) of them. Of these patients, seventeen were homozygous for L750V; five were compound heterozygous for L750V and c.2810+2T>G, another previously reported mutation in Mexican

patients (104); one patient was compound heterozygous for L750V and a large deletion in the promoter region, c.-6831_-39-1586del, that leads to the absence of transcripts from the allele carrying it (80); one patient was compound heterozygous for L750V and c.2471+1G>A, p.Leu825* (L825*); one patient was compound heterozygous for L750V and a novel premature stop-codon mutation c.1959G>A, p.Trp653* (W653*); and one patient was homozygous for c.1168del p.Arg390Alafs*26 (R390Afs*26) (92). Five patients (16.1%) did not have any pathogenic or likely pathogenic variant in NLRP7 and were screened for KHDC3L, but none of them had any mutation.

In the light of the high frequency of L750V in the 31 patients, we screened the record of the Pathology Department of the Instituto Nacional de Perinatologia for cases of RHMs since 2003. We found seven archived FFPE molar tissues, from seven additional unrelated patients that were available for analysis. DNA extraction from the chorionic villi of these tissues and their genotyping demonstrated that six are diploid biparental and one is diploid androgenetic monospermic. We next tested the six biparental moles for the presence of the founder L750V mutation. We found that two molar tissues were negative for L750V, three were heterozygous for L750V, and one was homozygous for L750V (Table1). The latter observation indicates that the father of the HM carries the L750V, known to be present in 5% of control subjects from the general Mexican population (78).

We next reviewed the results of all Mexican patients with RHMs who were referred from various hospitals and medical centers from the USA or Mexico to the RI-MUHC since 2006 for *NLRP7* and *KHDC3L* mutation analyses. We found 13 unrelated patients of them 12 had bi-allelic mutations in *NLRP7* (Table1). Seven were homozygous for L750V; one was compound

heterozygous for L750V and another previously reported promoter region deletion, c.-13413_2982-344del (90); two were compound heterozygous for L750V and c.2810+2T>C (90); one was compound heterozygous for L750V and c.2471+1G>A, p.L825*; and one was compound heterozygous for p.Tyr872* (Y872*) and c.2810+2T>C.

The states of origin of 44 unrelated patients analyzed on DNA from blood or molar tissues in this study or in Estrada et al. (78) with at least one copy of the L750V were available and are provided on the Mexican map in Fig.1, which shows an important clustering of these patients in the state of Mexico City where they were recruited and also in some neighboring states. Haplotype analysis of all the SNPs and variants that are covered by our Sanger sequencing demonstrated the inheritance of the L750V mutation on a shared haplotype between patients from various Mexican states (Table2), from rs775886 to rs269933 spanning 18,296 bp. We note that the shared haplotype is certainly larger; however, in Table2, we included only the single nucleotide polymorphisms that are covered by our Sanger sequencing.

Since *NLRP7* is highly rich in Alu repeats and so far, nine of its 80 reported mutations are mediated by Alu recombination (https://infevers.umai-montpellier.fr/web/), which can be easily missed when using only Sanger sequencing, we attempted to retrieve archived FFPE tissues from patients with no mutations to re-evaluate the diagnosis of their HMs and determine whether they are diploid biparental. Among the patients who were recruited in Mexico, we were able to retrieve four products of conception (POCs), two from each of patients 29 and 30. Morphological and genotypic evaluation of two POCs from patient 29 demonstrated that one is a triploid dispermic PHM and the other lacked morphological features of molar pregnancies and we

revised its diagnosis to miscarriage (Table1). Multiplex microsatellite genotyping of this miscarriage demonstrated its diploid biparental genome and SNP microarray confirmed the diagnosed and demonstrated the absence of aneuploidy (18). Therefore, this patient did not have RHMs (Table1). The two POCs from patient 30 fulfilled the morphological diagnosis of CHM and both were found diploid androgenetic monospermic by multiplex microsatellite genotyping. From a third patient, 31, no tissues could be retrieved, but one of her POCs had been karyotyped and found to be tetraploid 92,XXYY. Of the patients referred to the RI-MUHC, only one patient was negative for *NLRP7* mutations and four of her molar conceptions were available for genotype analysis and were found diploid androgenetic monospermic. This patient was later analyzed by exome sequencing and found to have bi-allelic mutation in *MEI1* (3). Therefore, the data on the POCs of these four patients explain the absence of *NLRP7* mutations in them since bi-allelic *NLRP7* mutations are associated with RHMs that are diploid biparental (Table1). In conclusion, of the five patients with no *NLRP7* mutations, only four had RHMs, which brings the number of patients with RHMs recruited in Mexico to thirty and the total number of analyzed and reviewed patients in this study to forty-three.

Discussion

Recurrent molar pregnancy is a rare disease. However, in the current study along with that of Estrada et al. (78), we report on a total of 70 unrelated patients with RHMs of Mexican origin (30 recruited in Mexico, 13 referred to the RI-MUHC, 7 molar tissues, and 20 reported in Estrada et al.). To our knowledge, this is the largest series from a single country and suggests a higher frequency of RHMs in Mexico than in other countries. This finding is in line with a previous report describing a higher frequency of RHMs in Mexico as compared to western countries.

Here, we describe the results of mutation analysis on 30 new unrelated patients with RHMs recruited in Mexico, seven molar tissues from seven unrelated patients with RHMs, and review mutation analysis on 13 unrelated patients of Mexican origin referred to the RI-MUHC. Of the 43 analyzed patients, excluding the molar tissues, L750V was present in homozygous or heterozygous state in 37 (86%) of them (Table3). These data make the L750V the most frequent *NLRP7* mutation reported to date and are in agreement with its presence at a minor allele frequency (MAF) of 0.025 in control subjects from Mexico (78) and 0.00310 in Latino population reported in gnomAD v2.1.1 (135 out of 35,430) (gnomAD (broadinstitute.org)) and Varsome (3 out of 848) (Varsome The Human Genomics Community).

In addition, this study revealed a second founder variant, c.2810+2T>G in the Mexican population that was present in eight unrelated patients (Table3). This mutation is also reported in databases with a MAF in Latino population of 0.0002892 (10 out of 34,574) in gnomAD v.2.1.1 and 0.0004 in Varsome. Of note, that L750V and c.2810+2T>G both appear to be specific for Mexican/Latino population (Varsome) and have never been reported in patients with RHMs or healthy subjects from other populations. However, the c.2471+1G>A mutation has been reported in patients of Pakistani, Indian, and Chinese origin, and this study revealed its presence for the first time in two unrelated Mexican patients, which is not unexpected since the Mexican population consists of a mixture of Native American inhabitants (56.4%), European migrants (41.8%), and West Africans (1.8%) (107). Ruiz-Linares et al. (108) estimated individual ancestry proportions in different countries from Latin America and found that in the Mexican population, Native American ancestry is highest in the center/south of the country where the highest number

of patients with L750V was observed. This suggests that L750V may have been inherited from the Native American population that remains to be demonstrated in future studies.

Two patients (98) and (106), the first with a homozygous L750V and the second with L750V and c.2810+2T>G, had each a live birth from a spontaneous conception that led to healthy children. These observations are in agreement with previous ones documenting the occurrence of a total of 13 live births (29,73,75,76,80,90), observed mostly in patients with mutations that have mild functional consequences on the protein such as missense, splice, or sometimes protein-truncating mutations at the end of the protein (75). Among the 13 reported live births, 12 children were reported to be healthy and only one was reported with various morphological abnormalities (76). Despite these relatively encouraging outcomes, spontaneous live births from such patients are extremely rare and account for approximately 1.5% of all their conceptions (1). Because the primary defect in patients with bi-allelic NLRP7 mutations is in their oocytes, ovum donation has been proposed to these patients as their best reproductive option. To date, eight such patients, including three reported in this study, patients 733, 1224, and 1878, and another in a patient that we previously reported in Estrada et al. (78), have achieved successful pregnancies from donated ova and conceived ten healthy live births (75,77,79).

Based on the above data and the replicated strong founder effect for L750V, if Sanger sequencing were to be used for mutation analysis, we propose to begin the analysis by sequencing exon 6 of *NLRP7*. If the patient is negative for the common mutation, completing the gene sequencing is then recommended. Genetic counseling of patients with bi-allelic *NLRP7* mutations must consider the age of the patients, the risk of neoplastic degeneration, which

occurred in 29% of the 31 patients recruited in Mexico, the scarcity of spontaneous live births in these patients, and the benefit of oocyte donation. Spontaneous live births have been observed in 13 patients; however, we still do not know if these children are at a higher risk for imprinting disorders. It is therefore important to keep in mind that the earliest known defect in patients with bi-allelic *NLRP7* mutations is the impaired establishment of maternal methylation marks in their oocytes. In addition, bi-allelic mutations in another member of the subcortical maternal complex, *PADI6*, which have been shown to cause female infertility, early embryonic arrest during preimplantation development (93), and miscarriages and HM (2,80) were recently documented in patients with Beckwith-Wiedemann and Silver-Russell syndromes (68,94). Therefore, a close follow-up of the pregnancies of patients with bi-allelic *NLRP7* mutations is highly recommended and may help monitoring for imprinting disorders which may lead to a broad spectrum of clinical manifestations.

Author contribution

All authors contributed to the study conception and design. Material preparation was performed by Maryam Rezaei, Irma Monroy, Mechtouf Nawel, Javier Pérez, Elsa Moreno, Yolotzin Valdespino, Carolina Galaz, and Guadalupe Razo. Data collection was performed by Monica Aguinaga, Carolina Galaz, Daniela Medina D, Raúl Piña, and Rima Slim. Analyses were performed by Maryam Rezaei, Irma Monroy, Mechtouf Nawel, Javier Pérez, and Guadalupe Razo. The first draft of the manuscript was written by Mónica Aguinaga and all authors commented on previous versions of the manuscript. Rima Slim revised the work critically for important intellectual content and approved the version to be published. All authors read and approved the final manuscript.

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Data Availability

All data and materials are available upon request.

Code availability

Not applicable.

Declarations

Ethics approval

The study was approved by the Instituto Nacional de Perinatologia (INPer) Review Board, study number: 212250-3220-11108-01-14 and the McGill Institutional Review Board (IRB# A01-M07-03A). This study was performed in line with the principles of the Declaration of Helsinki.

Consent to participate

All patients provided written consent to participate in our study.

Conflict of interest

The authors declare no competing interests.

Supplementary table 1.

Figures

Fig. 1 Geographical distribution of patients carrying L750V in Mexican states. The numbers refer to unrelated patients.

Tables

Table 1. Recapitulation of data on 71 analyzed patients with RHMs from Mexico					
Case N.	Patient ID	Reproductive history (complications)	*NLRP7* mutations	Complication	References
Mexican patients recruited in Mexico between 2013-2020					
1	ACC	6 PHM	L750V hom		
2	CEA	4 HM	L750V hom		
3	BBL	2 HM, END (preeclampsia), LB	L750V hom	GTD	
4	MMN (consanguinity)	4 HM	L750Vhom		
5	GHR*	3 HM, MC	L750V hom	GTD	
6	VGDE*	2 HM	L750V hom		
	VGLE (sister)	HM	L750V hom		
7	DJEY*	3 HM	L750V hom		
	DJER (sister)	2 HM	L750V hom		
	DJEG (sister)	2 HM	L750V hom		
8	OLO*	2 HM, MC	L750V hom		
9	CLL	2 HM	L750V hom	GTD	
10	RLMC	2 HM	L750V hom		
11	PQRM	3 HM	L750V hom		
12	PAF	4 HM, MC	L750V hom		
13	GGE*	4 HM	L750V hom	GTD	
14	DSL	3 PHM, MC	L750V hom		
15	VOM	3 HM	L750V hom		
16	CRA	HM, 2 MC	L750V hom	GTD	
17	ABH	5 CHM	L750V hom	GTD	
18	GEM	2 PHM	L750V, c.2810+2T>G		
19	MADM	HM, CHM, 2 MC	L750V, c.2810+2T>G		
20	CR	5 HM, LB	L750V, c.2810+2T>G	GTD	
21	PVI	2 HM, MC	L750V, c.2810+2T>G		
22	RJG	2 PHM, MC	L750V, c.2810+2T>G		
23	HME	HM, CHM, 2 PHM	L750V, c.-6831_-39-1586del	GTD	Rezaei et al, in preparation
24	RGR*	HM, 2 CHM, MC	L750V, c.2471+1G>A		
25	LCMV	HM, 2 PHM	L750V, W653*		
26	TGR*	2 HM	R390Afs*26 hom		Nguyen et al., 2018
27	GBNA	CHM, PHM	No mutation		
28	VPA	2 HM, LB	No mutation		
29	QVSL	PHM (triploid dispermic), PHM revised to MC	No mutation		
30	MCV	2 CHM (2 androgenetic monospermic), MC	No mutation	GTD	
31	MTMC	2 PHM, 3 MC, MC (92,XXYY)	No mutation		

Table 1. Recapitulation of data on 71 analyzed patients with RHMs from Mexico

HM, hydatidiform mole, which is used when the pathology report did not specify the classification; CHM, complete hydatidiform mole; PHM, partial hydatidiform mole; MC, miscarriage; END, early neonatal death; LB, live birth; GTD, gestational trophoblastic disease; BO, blighted ovum; IVF, in vitro fertilization; PGD, preimplantation genetic testing; HAT, total hysterectomy; hom, homozygous; het, heterozygous.

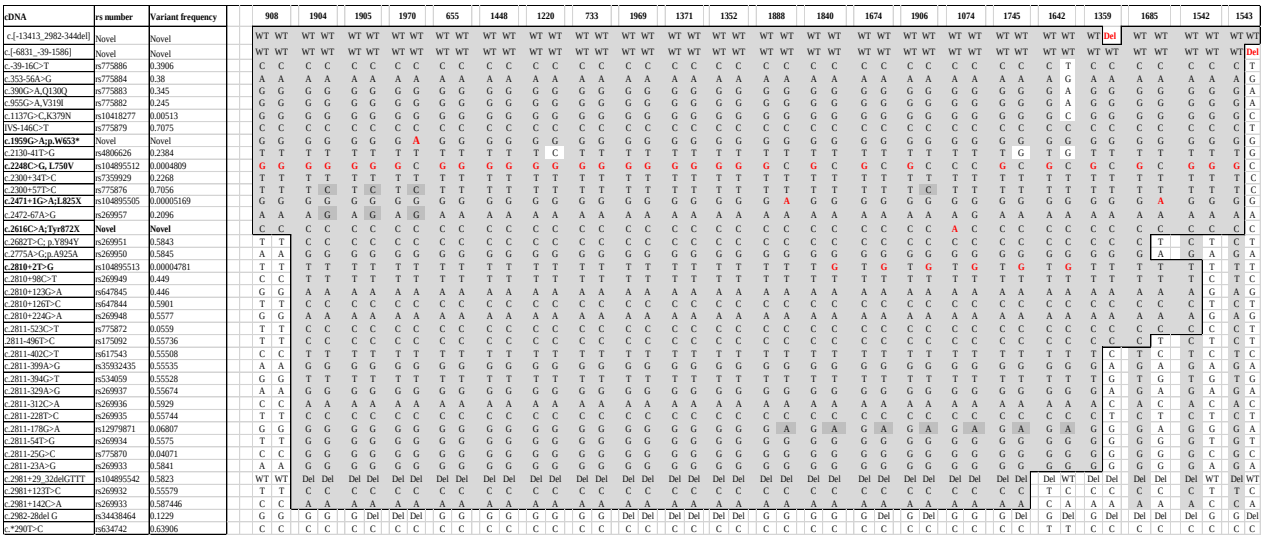

Table 2. Shared haplotype between patients

MAF minor allele frequency in gnomAD database. In column cDNA, bold character indicates pathogenic variants

Table 3. Distribution of mutations in 43 Mexican patients with RHMs	
Biallelic *NLRP7* mutations	Number of patients
L750V homozygous	24 (55.8%)
L750V, c.2810+2T>G	8 (18.6%)
L750V, c.2471+1G>A	2 (4.6%)
L750V, c.-13413_2982-344del	1 (2%)
L750V, c.-6831_-39-1586del	1 (2%)
L750V,W653*	1 (2%)
Number of patients with ≥ 1 L750V	**37 (86%)**
Y872X, c.2810+2T>G	1 (2%)
R390Afs*26 homozygous	1 (2%)
RHMs and no mutations in *NLRP7*	4 (9.3%)
Biallelic *MEI1* mutations	1 (2%)
Number of patients with RHMs	43

Table 3. Distribution of mutations in 43 Mexican patients with RHMs

Supplementary files

Supplementary table 1: Primers for amplification and Sanger sequencing of 11 exons of *NLRP7*						
Exon number	Amplification primer name	Amplification primer sequence	Annealing temperature in Celsius	Product size in bp	Sequencing primer name	Sequencing primer sequence
Exon 1	Ex1a	GCCCAATTACAGCCAAATCCCTGAG	66	606	Ex1a	
	Ex1b	GGCCGAGGCAGACAGATTACCTAAA			Ex1-rev2	TCCTTCCAGCATCCTCGCAC
Exon 2	Ex2-fwd	ACCGTGCTGGGCCAGATTTTCAGT	66	1152	Ex2-fwd	
	Ex3-rev	GCAGAGGTTGCAATGAGCAGAGACG			Ex2 rev2	ATGACCAGGACACCCCAGGTTCTA
Exon 3	Ex2-fwd	CACCTTGCATGCTCTCAAACACCA	66	1152	Ex3-fwd	ACCGTGCTGGGCCAGATTTTCAGT
	Ex3-rev	GCAGAGGTTGCAATGAGCAGAGACG			Ex3 rev2	CACCTTGCATGCTCTCAAACACCA
Exon 41	Ex4-1 fwd	GTAGTGGCTCCGTCTCTGCTCATTG	66	1550	Ex4-1 fwd	
	Ex4-3 rev	CGAGGCCGAATAAGAAGTGTCCTAC			Ex4-2 fwd	GACGACGTCACTCTGAGAAACCAAC
Exon 42	Ex4-4 fwd	GTGGGCGCAGATGTCCGTGTTC	66	805	Ex4-4 fwd	
	Ex4-4 rev	CCTAATTGCCAAGTCGTGTCTCC			Ex4-4 rev	
Exon 5	Ex5-fwd	GGTCTCAGTTTCTAGCCCAAGTT	62	839	Ex5-fwd	
	Ex5-rev	ACACGGTGAAAACCTGTCTGTGC			Ex5-rev2	CAAGAAGCTTAGTCATCGTT
Exon 6	Ex6WF	CCCGGCCAAGAACTTCTAAT	62	499	Ex6WF	
	Ex6WR	ACGGCATCTGGAGTGGTTAC			Ex6WR	
Exon 7	Ex7-fwd	GATCACGCCTTTGCATTCCAGACTG	62	471	Ex7seq	AGCTGATAGGGTATACTCTG
	Ex7-rev	AGCAGGTGTTTATTTCAGCAAGAGG			Ex7-rev	
Exon 8	Ex8f2	TGGCCATGATGACTCCCACAGG	62	418	Ex8f2	
	Ex8r2	CCAGGTTTTTAAAAGTTACATTTG			Ex8r2	
Exon 9	Ex9fwd2	GCTTCACAGGGCGTTAGCCAGAGG	62	550	Ex9fwd2	
	Ex9rev2	TAAAAATGAGGCCAGGCATGATGG			Ex9rev2	
Exon 10	Ex10fwd	GCCCCAGCAAATATGCTGCTTG	66	1022	Ex10a 1	AAGGTGCTGGGGCTACAGGTGTCT
	Ex10rev	GGACATGTTGGCATGCCTCTAG			Ex10a seqr	AAACCCATACCTGAGTAT
Exon 11	Ex11(11)-fwd	TGTCCCCCAGAAAATCCCAAAAAC	62	602	Ex11(11)-fwd	
	Ex11 (11)-rev	ATGGCGCCTGAGTTAGGGAAGAAA			Ex11 (11)-rev	
Because NLRP7 introns are highly rich in repeat elements and stretches of mononucleotides, for some exons, additional sequencing primers were used to avoid sequencing through stretches of mononucleotides. These primers are listed on the right.						

Supplementary Table 1: Primers for amplification and Sanger sequencing of 11 exons of NLRP7

PREFACE TO CHAPTER 3

In order to find a second patient with mutation in any of the identified candidate genes in aim 2, we initially performed targeted sequencing on 96 patients with milder phenotypes (patients with 1 mole and at least 2 miscarriages or patients with at least 3 recurrent miscarriages). There are two reasons to include patients with recurrent miscarriages. 1) Bi-allelic mutations in the three meiotic genes, *MEI1*, *TOP6BL* and *REC114*, were associated also with recurrent miscarriages, early embryonic arrest after ART, secondary infertility after RHM, infertility in our patients and/or other studies reported after their identification (3). 2) Bi-allelic mutations in *PADI6* were first described in infertile patients and later we reported a patient with one HM phenotype and several miscarriages and my work in chapter two described a second patient with four molar pregnancies of which two showed some features of genetic imprinting disorders such as BWS and SRS (2).

We submitted 96 samples for targeted sequencing in February 2019 for nine of our best candidate genes (*PADI6, TLE6, EZR, KHDC3L, FBXO22, MICAL1, MYLK3, SHGB, PDE3A*). However, unfortunately, we did not find any second hit in our candidates, which indicates the high genetic heterogeneity of this condition. So, we decided to use WES directly. While performing these exomes, another member of the lab found a homozygous splice site mutation in *CCNB3* gene in a patient with 16 RMs. *CCNB3* had been found in 2018 as the cause of recurrent miscarriages for the first time (4). Furthermore, we showed that one of the patient's miscarriages is triploid digynic resulted from the failure of meiosis 1.

Chapter 3

A protein-truncating mutation in *CCNB3* in a patient with recurrent miscarriages and failure of meiosis I

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Conflict of Interest Statement

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Recurrent miscarriage (RM) is defined by the occurrence of at least two pregnancy losses prior to 22 weeks of gestation and affects up to 5% of couples trying to conceive (109–111). RM has a significant emotional impact on couples and the repetitive nature intensifies the grief experienced. A recessive missense in cyclin B3 (*CCNB3*) has recently been shown in two sisters with RM and triploidy of maternal origin (4). Here, we report a novel recessive *CCNB3* mutation, c.4091+1G>A, p.Val1321Glyfs*4, in a patient with 16 RM and show that one of her miscarriages is triploid digynic resulted from the failure of meiosis I.

RM is clinically and genetically highly heterogeneous. After comprehensive clinical and laboratory testing, in 50% of couples, no abnormalities are identified, and such cases are categorised as RM of unexplained clinical aetiology. To date, little is known about their genetic causes, and known genes explain only a minority of cases. One of the many factors that have hampered our understanding of the genetics of recurrent miscarriages is their complexity, genetic heterogeneity and the difficulties in homogenising these entities to simplify their studies. In many cases of recurrent miscarriages of unknown clinical aetiology, it is impossible to know whether the defect originates from the male or the female, and whether it is in a dominant or a recessive state. Also, it is impossible to know if the defect is transmitted from the parents to the miscarried conception or if it occurred de novo in the miscarriage. While the germline origin of male causes of miscarriages could be sometimes diagnosed based on semen analysis, diagnosing the origin of female causes of miscarriages is more challenging; in many cases, it is impossible to distinguish germline from uterine or systemic defects. Consequently, despite the use of next-generation sequencing, which has greatly facilitated the identification of many causative genes for male infertility (112), few genes responsible for recurrent miscarriages have been identified.

The proband (ID 1264) has had 16 miscarriages of unexplained clinical origin over a period of 15 years with the same partner. The first miscarriage occurred in her late 20s. She and her partner had normal karyotypes and were in good health. They consulted several specialists and had undergone complete evaluations in different countries, cities and medical centres. The only detected abnormality was a positive antiphospholipid antibody in the patient based on which, she was believed to have a postimplantation immunological aetiology underlying her recurrent pregnancy loss and was then treated with low dose aspirin and low molecular weight heparin. Because of continued miscarriages despite standard empirical treatments including paternal leucocyte immunotherapy, intravenous infusion of immunoglobulin and prednisone were tried as well as empirical infertility treatment. Intrauterine insemination was tried 3–4 times and led to only chemical pregnancies with a modest rise in beta-hCG that dropped soon after. All her spontaneous conceptions ended in miscarriages between 6 and 7 weeks of gestation except for one that lasted for 8 weeks. Two pregnancies were karyotyped using classical culture-based cytogenetics method and had 46,XX karyotype, which may have originated from maternal cell contamination. In vitro fertilisation and preimplantation genetic testing for aneuploidies were considered, but by the time she was referred to the McGill Reproductive Centre, she was already in her mid-40s with diminished ovarian reserve.

In an attempt to identify a potential genetic aetiology of her recurrent miscarriages, we performed whole exome sequencing on her blood DNA and analysed the data for recessive defects. We considered homozygous or compound heterozygous variants with minor allele frequency <0.005 in Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org) that were located in the coding regions (missense, nonsense, frameshift, indels) or affected canonical splice sites. This led to the identification of a novel

homozygous variant that is not listed in gnomAD, NM_033031.3:c.4091+1G>A, and that affects the splice donor site of exon 12 of cyclin B3 (*CCNB3*) gene (figure 1). This variant is located in a large run of homozygosity of approximately 70 Mb and is predicted to be pathogenic according to the American College of Medical Genetics and Genomics guidelines using Varsome (https://varsome.com/) (113). *CCNB3* had recently been reported by Fatemi et al, to underlie the causation of recurrent miscarriages in two sisters and was therefore the most attractive candidate. Assessing the impact of this variant on CCNB3 splicing demonstrated the skipping of exon 12 (figure 1), which leads to a frameshift and truncation of the protein after 4 amino acids, p.Val1321Glyfs*4, in the highly conserved cyclin C-terminal domain (from amino acid 1259 to 1375).

Only one formalin-fixed paraffin embedded product of conception (POC) from this patient was available. Its morphological evaluation confirmed the diagnosis of miscarriage (figure 2A). Multiplex microsatellite genotyping using the PowerPlex 16 HS System (Promega, Fitchburg, Wisconsin, USA) on DNA extracted from the chorionic villi along with parental DNA demonstrated triploidy of maternal origin based on the presence of two different maternal alleles in the POC at three informative markers (figure 2B). The triploidy was also confirmed by FISH (figure 2C). Using pericentromeric microsatellite markers, mapped at <5 Mbp from the centromeres, we demonstrated that the maternal triploidy resulted from the failure of homologous chromosome separation at meiosis I (figure 2D).

CCNB3 is highly conserved during eukaryote evolution and plays a critical role in female meiosis. Like other B-type cyclins (cyclin B1 and B2), *CCNB3* binds to CDK1 via its cyclin domains, and the two proteins form a complex that phosphorylates the anaphase-promoting complex/cyclosome (APC/C) allowing therefore the initiation of anaphase I and the separation of

homologous chromosomes. In Drosophila melanogaster, females deficient for Ccnb3 are infertile due to the inability of their oocytes to complete meiosis I (114,115). In mice, Ccnb3 knockout also causes female infertility due to the failure of metaphase to anaphase transition in meiosis I and the extrusion of the first polar body. The infertility in these mice appeared to be due to embryonic lethality before embryonic day 7.5 and some of their oocytes fertilised by intracytoplasmic sperm injection led to triploid embryos (116). Data on these two animal models are in agreement with our data on the analysed triploid POC from our patient, which also resulted from a failure to complete meiosis I. To date, only two sisters with recurrent miscarriages and recessive defect in *CCNB3* have been reported by Fatemi et al, and two of their POCs were characterized and found to be triploid digynic due to the failure of meiosis II (4). The discrepancy between the meiotic origin of the maternal triploidy in our case and those reported by Fatemi et al (4) may be due to severity of the mutation in our patient (protein-truncating), which may have led to an earlier arrest of meiosis than the milder mutation (missense) reported by Fatemi et al (4). The latter may have been more permissive for meiotic progression to meiosis II.

Reproductive failure of female origin manifests mainly in three forms, recurrent hydatidiform moles, infertility and recurrent miscarriages. The use of exome sequencing in the past decade has accelerated the identification of causative genes for recurrent hydatidiform moles and female infertility. However, because of the challenges outlined above, much less success has been achieved in identifying causative genes for recurrent miscarriages. The sad story of our patient illustrates the struggle of patients with RM to conceive and that of the physicians managing them including often the use of many empirical and/or ineffective treatments. Our report highlights the benefit of performing clinical exomes on such patients despite the knowledge that the diagnostic

yield may be low and that this approach may not explain the genetic aetiology of the majority of cases.

Ethics statements

Patient consent for publication

Not required.

Acknowledgments

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Footnotes

Contributors MR did the experiments and contributed to the writing of some sections of the manuscript. RS completed the writing and supervised the work. WB referred the patient and contributed to the writing of the clinical description section. US performed the FISH analysis. EB and JM filtered raw data of exome sequencing.

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Competing interests: None declared.

Provenance and peer review: Not commissioned; externally peer reviewed.

Figures

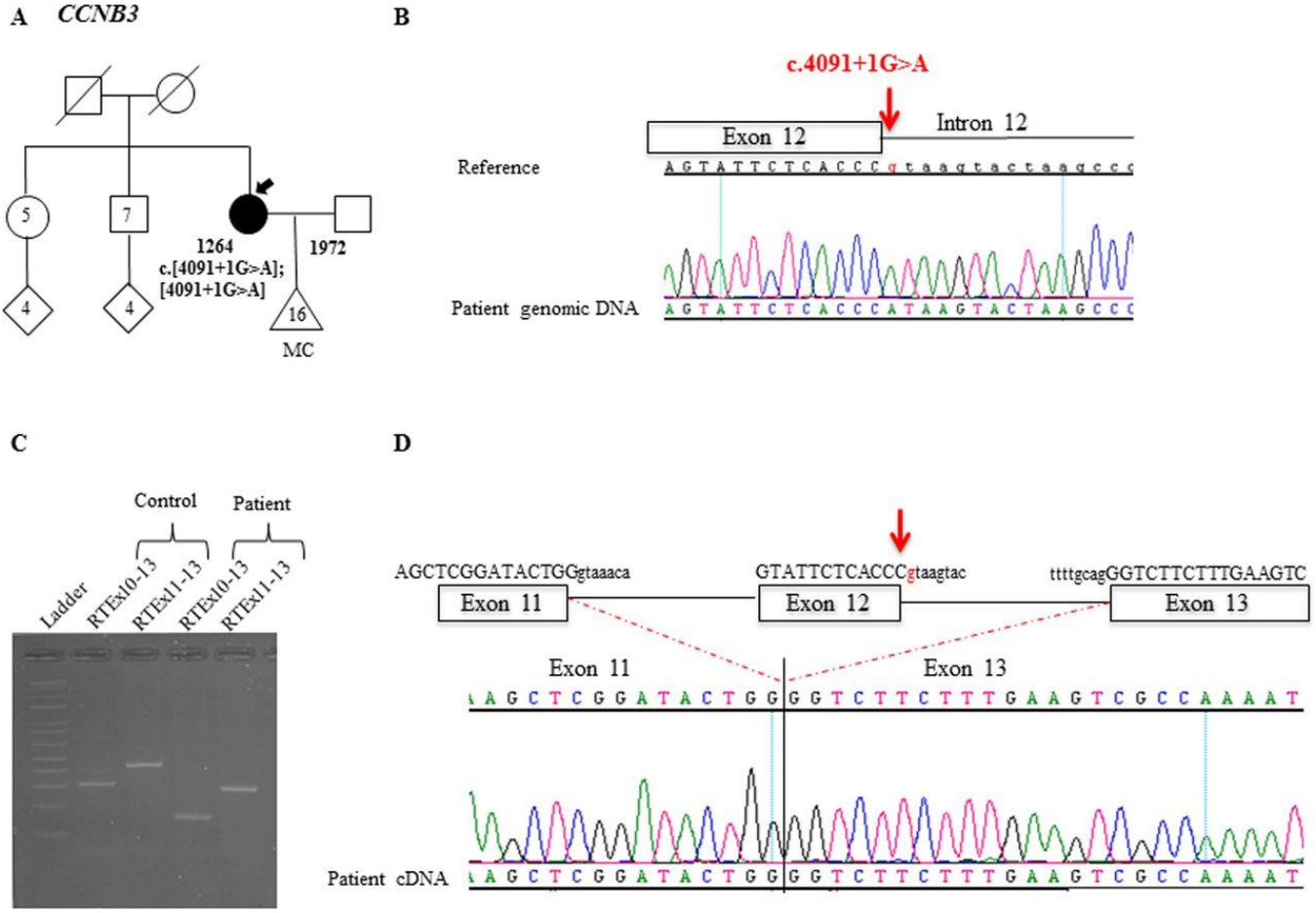


Figure 1. Pedigree and chromatograms of patient 1264 with a homozygous mutation in the canonical splice donor site of exon 12 in CCNB3 gene. (A) and (B) Pedigree and chromatogram of genomic DNA. (C) and (D) RT-PCR from the patient and control subject with two pairs of primers located in exons 10 and 13 (359 bp) or 11 and 13 (284 bp) showed shorter cDNA fragments in the patient. Chromatogram of cDNA shows the deletion of exon 12.

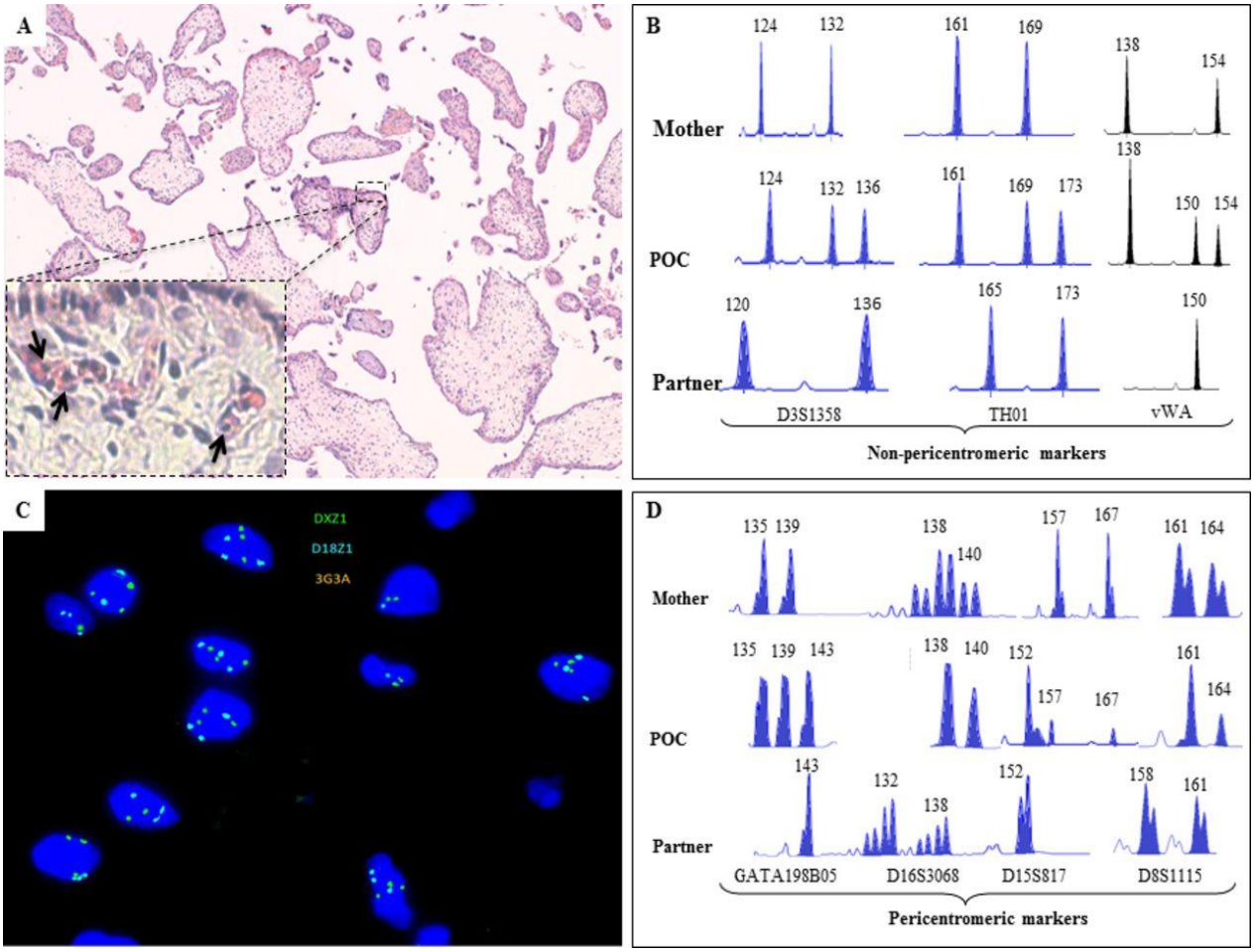


Figure 2. Morphology and genotyping results of one product of conception (POC). (A) A microphotograph of the POC showing chorionic villi with abnormal morphology but without trophoblastic proliferation. The inset shows a magnification of fetal vessels with nucleated red blood cells. (B) Microsatellite genotyping results demonstrate a triploid miscarriage of maternal origin. (C) FISH with centromeric probes from chromosomes X, Y and 18 confirmed the triploidy of the POC and showed the presence of three X chromosomes. 3G3A stands for three gonosomes and three autosomes. (D) Genotyping of four pericentromeric markers demonstrated that the triploidy resulted from the failure of meiosis I.

PREFACE TO CHAPTER 4

When I started my PhD project, our lab had already started the project of identification of new genes for RHMs. In 2018, *MEI1*, *TOP6BL*, *REC114* were found as the causative genes for recurrent androgenetic moles. In line with the same goal, I started to look for other responsible genes for this phenotype with having the high genetic heterogeneity of this condition in our mind. For sure, one thing that is becoming clear for us day by day, is the fact that having a successful pregnancy from gametogenesis, fertilization and early embryonic development is a heterogeneous condition and it needs to be dissected in detail. In chapter 4, which is a manuscript in preparation, I describe the work of gene identification in patients with recurrent androgenetic HMs and reveal five more genes of which four have clear roles in meiosis I.

Chapter 4

Title: Identification of five new candidate genes for recurrent androgenetic moles

Abstract

Recurrent hydatidiform moles (RHM), is a condition in which women experience a rare complication of pregnancy with no embryo and excessive proliferation of the trophoblast. They have mostly androgenetic genomes with all the chromosomes originating from a haploid sperm and no maternal chromosomes.

Although androgenetic HMs have been described since 1977, their genetics and how they occur have been unknown till in 2018, it was reported that bi-allelic mutations in *MEI1*, *TOP6BL/C11ORF80* and *REC114*, cause recurrent androgenetic HMs. Furthermore, investigating the occurrence of androgenesis in Mei1-deficient mice has uncovered this meiotic defect in mammal's which is the extrusion of all maternal chromosomes and their spindles into the first polar body.

Continuing our search for causative genes for androgenetic RHMs, we have identified bi-allelic three protein truncating and one missense mutation in meiotic genes, *HFM1, MAJIN, SYCP2* and *TOPBP1*, respectively. The role of all these meiotic genes is to prepare chromosomes for an accurate homologous recombination and faithful chromosome segregation during meiosis I. Moreover, we found bi-allelic missense mutation in *FOXL2* which is an important transcription factor in ovary functions. The genotype of the molar tissues due to recessive mutations in these five genes have been demonstrated to be androgenetic.

Introduction

Hydatidiform mole (HM) (MIM:231090) is an abnormal human pregnancy characterized with abnormal embryonic development and excessive trophoblastic proliferation and usually is the product of a chromosomal abnormality with excessive contribution of paternal genome. HM occurs mostly as sporadic, non-recurrent with the incidence of 1 in every 600 pregnancies (1). Based on histological evaluation, 50% of HMs are partial HMs (PHMs) with triploid dispermic genotype and the remaining 50% are complete HMs (CHMs) with diploid androgenetic genotype (all the chromosomes are paternally derived from one sperm (monospermic in 85% of cases) or two sperms (dispermic in 15% of cases) and no maternal genome (18). Androgenetic CHM (AnCHM) occurs with the incidence of 1 in every 1,400 pregnancies (7) and they have been described since 1977, however, the genetic susceptibility and how/when the maternal genome is lost in these conceptions have remained as open questions since then. While the genetic causes of triploid dispermic moles is still unknown, the genetics of CHMs has started to become unraveled since 2018 that Nguyen et al, showed that mutations in *MEI1*, *TOP6BL* and *REC114*, with roles in double strand break formation during meiosis I, are the causes for androgenetic CHMs. By studying *Mei1*-deficient mice, this study proposed a plausible mechanism for the genesis of AnCHMs which is "the extrusion of all the oocyte chromosomes with their spindles into the first polar body" (3).

We performed whole exome sequencing (WES) on women with RHMs, miscarriages, and infertility, who were screened and negative for mutations in *NLRP7* and *KHDC3L*. We identified bi-allelic deleterious mutations in four meiotic genes including *HFM1* (Helicase for meiosis 1)

(MIM: 615684), *MAJIN* (Membrane-anchored junction protein) (MIM: 617130), *SYCP2* (Synaptonemal complex protein 2) (MIM: 604105), *TOPBP1* (DNA Topoisomerase II-binding protein 1) (MIM: 607760) and in *FOXL2* (Forkhead box transcription factor) (MIM: 605597) genes in six women including a familial case with two sisters affected by RHMs in *HFM1* case. In *TOPBP1* and *MAJIN* cases, recurrent miscarriages and infertility were reported in other family members. We demonstrated that their HMs have the histopathological features of CHMs and have androgenetic monospermic genomes. All five genes are conserved during evolution. While *HFM1*, *MAJIN*, *SYCP2* and *TOPBP1* are known to play roles during early homologous chromosome pairing and recombination in the oocyte (71,117–119), *FOXL2* functions in ovary and eyelid muscle development (120).

Material and Methods

Blood or saliva from patients and their family members were collected after obtaining the written informed consents from all participants and the study was approved by McGill University Research Ethics guidelines (Institutional Review Board # A01-M07-03A). Genomic DNA was isolated from whole blood cells using Flexigene DNA Kit (QIAGEN). The products of conception from different pathology laboratories were retrieved for genotype analysis. *NLRP7* and *KHDC3L* mutational screening were performed to exclude the presence of mutations in these two genes before sending for whole-exome sequencing. PCR conditions and the sequences of primers were previously described (29), and samples were sent for bidirectional Sanger sequencing.

500 ng peripheral blood leukocyte DNA from patients was captured with either Roche Nimbelgen SeqCap EZ Human Exomes or MedExomes capture kits and then sequenced with

paired-end 100 bp reads on Illumina HiSeq 6000. Sequence reads were mapped to the human reference genome (hg19) with Burrows–Wheeler Aligner (5) (V. 0.7.17), duplicate reads flagged using Picard (V. 2.27.4) and excluded from further analyses. Variants were called using GATK HaplotypeCaller (121) (V. 4.2.4.0) and mutations were annotated using both ANNOVAR (122) and custom scripts.

Annotated variants were filtered against the common germline polymorphisms present in dbSNP135, the 1000 Genomes project and Genome Aggregation Database (GnomAD) (v2.1.1) (123), keeping those coding (missense, nonsense, frameshift, indels or canonical splice site changes), with a maximum population minor-allele frequency (MAF) of less than 0.005 in GnomAD. Finally, only the most likely damaging variants (nonsense, canonical splice-site, conserved missense, and coding indels) were considered and manually checked in Integrative Genomics Viewer (IGV) (124) if they were predicted to be deleterious by at least two bioinformatics algorithms (Polymorphism Phenotyping v2 (PolyPhen-2), SIFT, MutationTaster, Combined Annotation Dependent Depletion (CADD) and assessed under the recessive mode of inheritance which is the mode of inheritance in all RHMs cases (Supplementary figure 2).

Result

Identification of bi-allelic mutations in four meiotic genes, *HFM1, MAJIN, SYCP2, TOPBP1* and *FOXL2*

In *HFM1*, by analyzing the exome sequencing of proband (1802) with three HMs who belongs to a familial case, a novel homozygous protein-truncating mutation c.312dupT (p.Tyr1042Leufs*7) was found (Figure 1A, B). The mutation was in a run of 25.07 Mb homozygosity (ROH) on chromosome 1 (supplementary figure 1). Analyzing additional samples from other family

members identified the same mutation in a homozygous state in her affected sister (with two HM) and in heterozygous state in the proband's parents who are consanguineous. Interestingly, the proband's maternal aunt, 1922, had experienced one HM after being diagnosed with infertility and later on she adopted a child was heterozygous for the proband's mutation (Figure 1A). The H&E staining of the only POC from patient 1802 revealed hydropic chorionic villous with excessive circumferential trophoblastic proliferation which is the characteristics of CHMs (Figure 1C). Additionally, genotyping of the same POC performed by the group of Dr Majid Fardaei showed a monospermic androgenetic CHM due to inheritance of alleles at 8 markers from the father in the POC at all the eight polymorphic microsatellite loci (Figure 1D).

In *MAJIN*, using exome sequencing in patient 1824 (Figure 2A B, D) with two molar pregnancies, a novel splice donor mutation, c.349+1G>T was found (Figure 2B, D), in a homozygous state in a 48.3 Mb of ROH on chromosome 11 (supplementary figure 1). The mutation segregated from both parents who were found to be heterozygous carriers. I investigated the effect of the invariant splice mutation c.349+1G>T in exon 6 using RT-PCR on RNA from Epstein Barr Virus–transformed lymphoblastoid cell lines (LCL) from the patient and controls. I found that the mutation leads to the absence of exon 6 and flanking exons (exon 5 + exon 6 + exon 7=221 bp size) in the patient compared to the control. *MAJIN* transcripts in the patient was not detected even by increasing the amount of total RNA in the reverse transcription reaction from 3 ug to 40 ug (Figure 2C). Characterization of two HM tissues from patient 1824 with bi-allelic mutations in *MAJIN*, by one of our lab members (Iqbal Sandhu) showed that the HM belongs to the CHM by morphology and P57KIP2 staining. Also, it was demonstrated that the 2 HM from patient 1824 have androgenetic monospermic genome by microsatellite genotyping (Figure 3A). Additionally, she was able to characterize one POC from the mother of

the patient 1824, who is heterozygous carrier of the splicing mutation in *MAJIN*, c.349+1G>T, and found that it is a PHM with a triploid dispermic genome. (Figure 3B).

In *SYCP2* case, the proband 1954, has experienced four recurrent CHMs after one year of primary infertility. After analyzing the patient's exome, another lab member identified a homozygous c.2530-2A>G was found. This change affects the canonical splice acceptor site of exon 27 (Figure 4A, B) in a 19.7 Mb ROH on chromosome 20 (supplementary figure 1). Only the patients' mother DNA was available, and it was found to be heterozygous carriers of the mutation. Unfortunately, we did not have access to the patient's blood to investigate the consequence of the mutation on the splicing at RNA level. However, using Human Splicing Finder (https://www.genomnis.com/access-hsf), which is an online bioinformatics tool to predict splicing signal alterations, it was predicted that c.2530-2A>G, abolishes the wild type of splice acceptor site and therefore most probably affects the splicing process.

Compound heterozygous variants in *TOPBP1* was found by another lab member (Figure 5A, 5B) in patient 1601 with a complex reproductive history including one androgenetic CHM, 2 miscarriages, 2 triploid digynic miscarriages, 1 termination of pregnancy at week 20 due to abnormal karyotype, ring chromosome 13, r(13)(p11.2q14.2), in amniocytes and finally in 2017, 2 live births with the help of ICSI. The two mutations segregated in the family; the variant c.488A>G (p.K163R) is conserved in human (PolyPhen = 0.7, CADD = 13.86), chimpanzee, orangutan, mouse, dog and elephant and is inherited from father and c.3007G>A (p.A1003T) is conserved in all above-mentioned vertebrates except mouse and dog (PolyPhen = 0.003, CADD = 11.85), and is inherited from her mother (Figure 5C). Of note, c.488A>G (p.K163R) is in BRCA1 C Terminus (BRCT) domain of TOPBP1 protein, which is found predominantly in proteins involved in cell cycle checkpoint and DNA damage response (Figure 5D). The genotype

of one of the 4 miscarriages was found to be triploid digynic and using microsattelite genotyping it was revealed that this conception is the result of maternal meiosis II failure (Figure 6A, 6C). Histopathology and microsatellite analysis of the only HM revealed its androgenetic monospermic genotype (Figure 6B. Of note, she had also a mosaic conception including diploid androgenetic and diploid biparental cells that was confirmed both by FISH and microsatellite genotyping analysis (Figure 6D).

Finally, in *FOXL2*, I found a missense c.500T>C, (p.F167S) in patient 1690 (Figure 7A) in a homozygous state in a ROH of 10.47 Mb on chromosome 3 (supplementary figure 1). The patient has experienced 5 androgenetic CHMs, 3 miscarriages and one stillbirth. p.F167S is conserved from human to opossum and fish (Figure 7B).

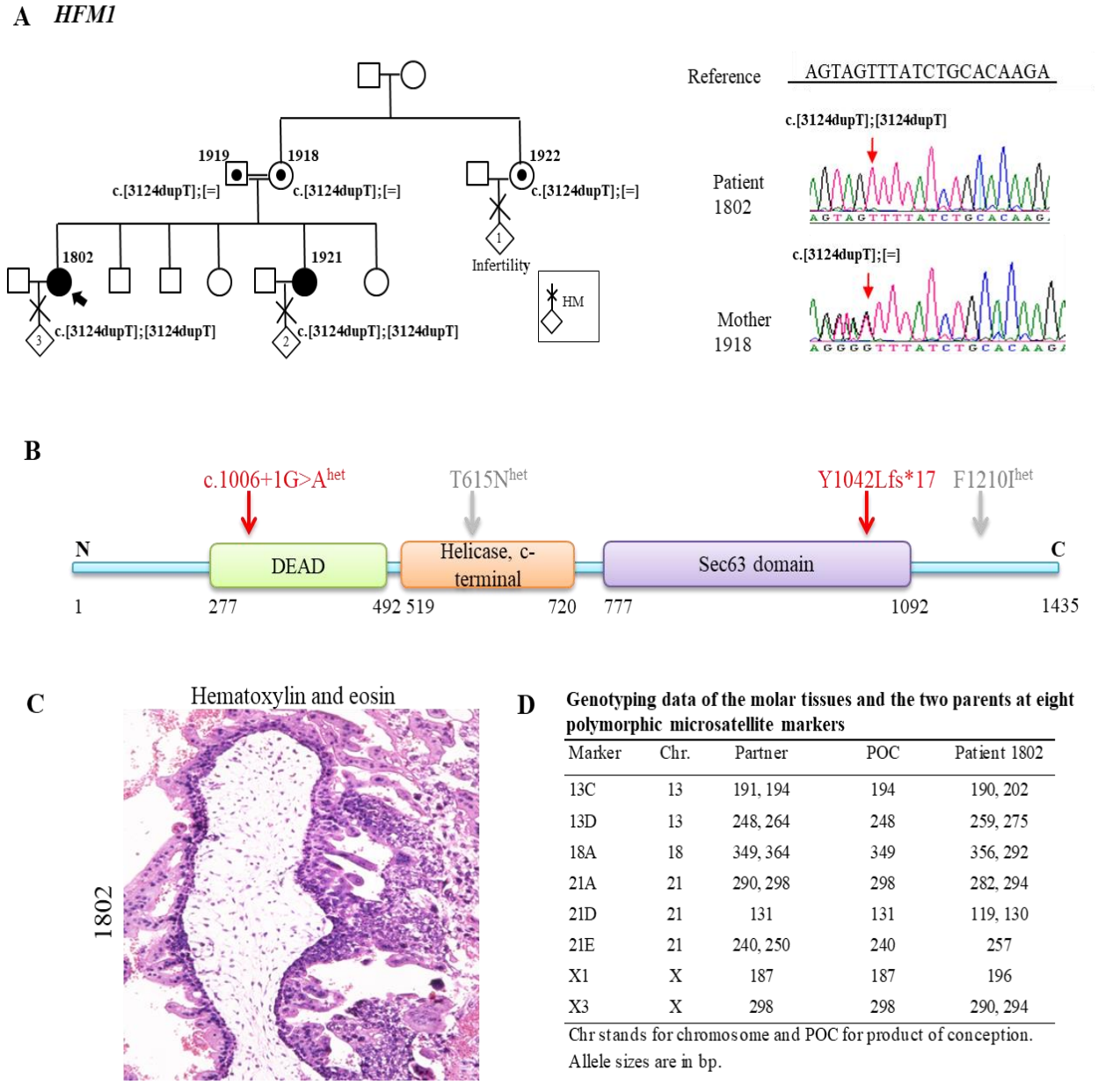
Discussion

In this study we identified bi-allelic mutations in five novel candidate genes, including four with roles in meiosis, *HFM1, MAJIN, SYCP2, TOPBP1* and one transcription factor, *FOXL2*, which could be responsible for androgenetic monospermic hydatidiform moles. This evidence is based on the identification of bi-allelic three protein truncating mutations in *HFM1* in a familial case, in *MAJIN* and *SYCP2*, each in one patient (each in 11-31 Mb runs of homozygosity), and 3 missenses in *TOPBP1* and *FOXL2* each in one patient. Strikingly, the four meiotic genes are evolutionary conserved, and all are required for homologous pairing and recombination during prophase I. Analyzing HMs from patients with mutations in all 5 genes demonstrated that they meet the criteria of complete HMs and have diploid androgenetic monospermic genomes in *HFM1*, *MAJIN*, *TOPBP1* and *FOXL2* cases. In the patient with bi-allelic mutation in *TOPBP1*, triploid digynic, and diploid mosaic conception including one diploid biparental and another

diploid androgenetic monospermic cellular populations were seen beside the single androgenetic monospermic HM. Taken together, these data indicate that these genes play role in the genesis of androgenetic CHMs. *Hfm1* and *Majin* null mouse mutants of both males and females are infertile due to blockage of meiosis 1 during spermatogenesis and oogenesis, respectively (117,118). In humans, variants in *HFM1* and *FOXL2* have been associated with premature ovarian failure, a condition in which ovarian function stops before age 40 and is characterized with oligomenorrhea or amenorrhea (120,125,126). In *SYCP2*, three variants in heterozygous state were reported in men with azoospermia (119). All the four meiotic genes are involved during prophase of meiosis 1 in order to have a proper homologous pairing and/or recombination.

In 2018, Nguyen et al reported bi-allelic mutations in *MEI1*, *TOP6BL*, and *REC114* in 5 unrelated women with recurrent androgenetic monospermic CHMs and miscarriages. Noteworthy, non-obstructive azoospermia has been reported in the case of *MEI1* and *TOP6BL*. Interestingly, these three genes also play a key role in the formation of meiotic DSBs during meiosis I (3). Altogether, we reported another five genes *HFM1*, *MAJIN*, *SYCP2* and *TOPBP1* with roles in prophase I and *FOXL2* an essential transcription factor for female reproduction which all putatively cause androgenetic HM. These findings may shed new lights on the mechanism(s) of androgenesis in recurrent HM pregnancies.

Figures



Genotyping data of the molar tissues and the two parents at eight polymorphic microsatellite markers

Marker	Chr.	Partner	POC	Patient 1802
13C	13	191, 194	194	190, 202
13D	13	248, 264	248	259, 275
18A	18	349, 364	349	356, 292
21A	21	290, 298	298	282, 294
21D	21	131	131	119, 130
21E	21	240, 250	240	257
X1	X	187	187	196
X3	X	298	298	290, 294

Chr stands for chromosome and POC for product of conception.
Allele sizes are in bp.

Figure 1. (A) Pedigree structure, Sanger validation, and segregation of the mutations in HFM1 in the family of proband 1802. (B) HFM1 protein structure with the location of the variants (Red variants have been observed in patients with HM phenotype and grey variants have been seen in patients with premature ovarian insufficiency. Het denotes mutations found as a single heterozygous mutation/variant, and hom those found in a homozygous state. (C) The

morphology of one HM from patient 1802 which shows a chorionic villous with excessive circumferential trophoblastic proliferation (arrows) typical of HM. (D) Genotype data of the same product of conception (POC) shown in C along with parental DNA, revealed one single paternal allele at each marker demonstrating the androgenetic monospermic genome of the mole.

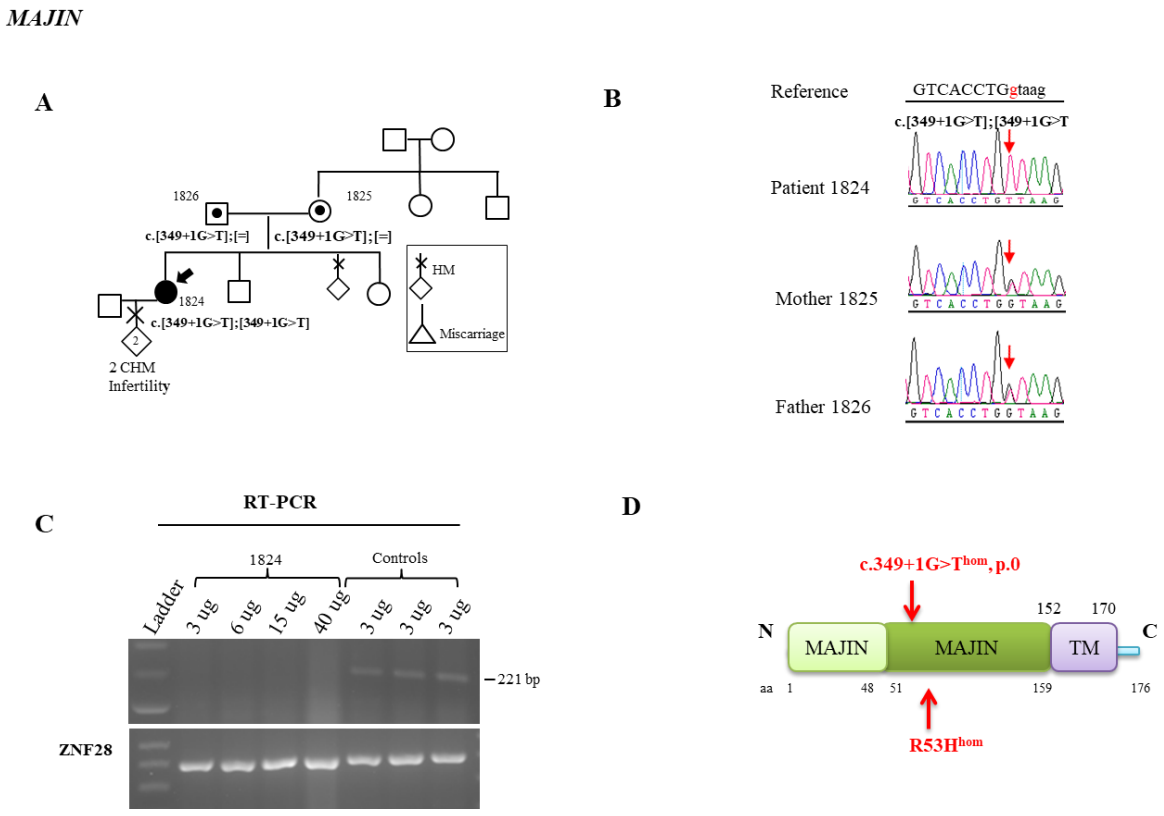


Figure 2. (A). Pedigree structure, (B) Sanger validation, and segregation of the mutation in *MAJIN* in the family of proband 1824. (C) The effect of the invariant splice mutation (c.349+1G>T) of exon 6 using RT-PCR on RNA from Epstein Barr Virus–transformed lymphoblastoid cell lines (LCL) from patient demonstrating the absence of (221 bp) i.e., Eexon6 (126 bp) and flanking exons Exon 5 (63 bp) and Exon 7 (32bp) in the patient at cDNA level compared to controls, even by increasing the amount of total RNA from 3 ug to 15 ug. (D) The protein structure along with the mutation in our patient (above the protein) and a recently

reported pathogenic missense in an azoospermic male (below the protein). Hom denotes mutations found in a homozygous state.

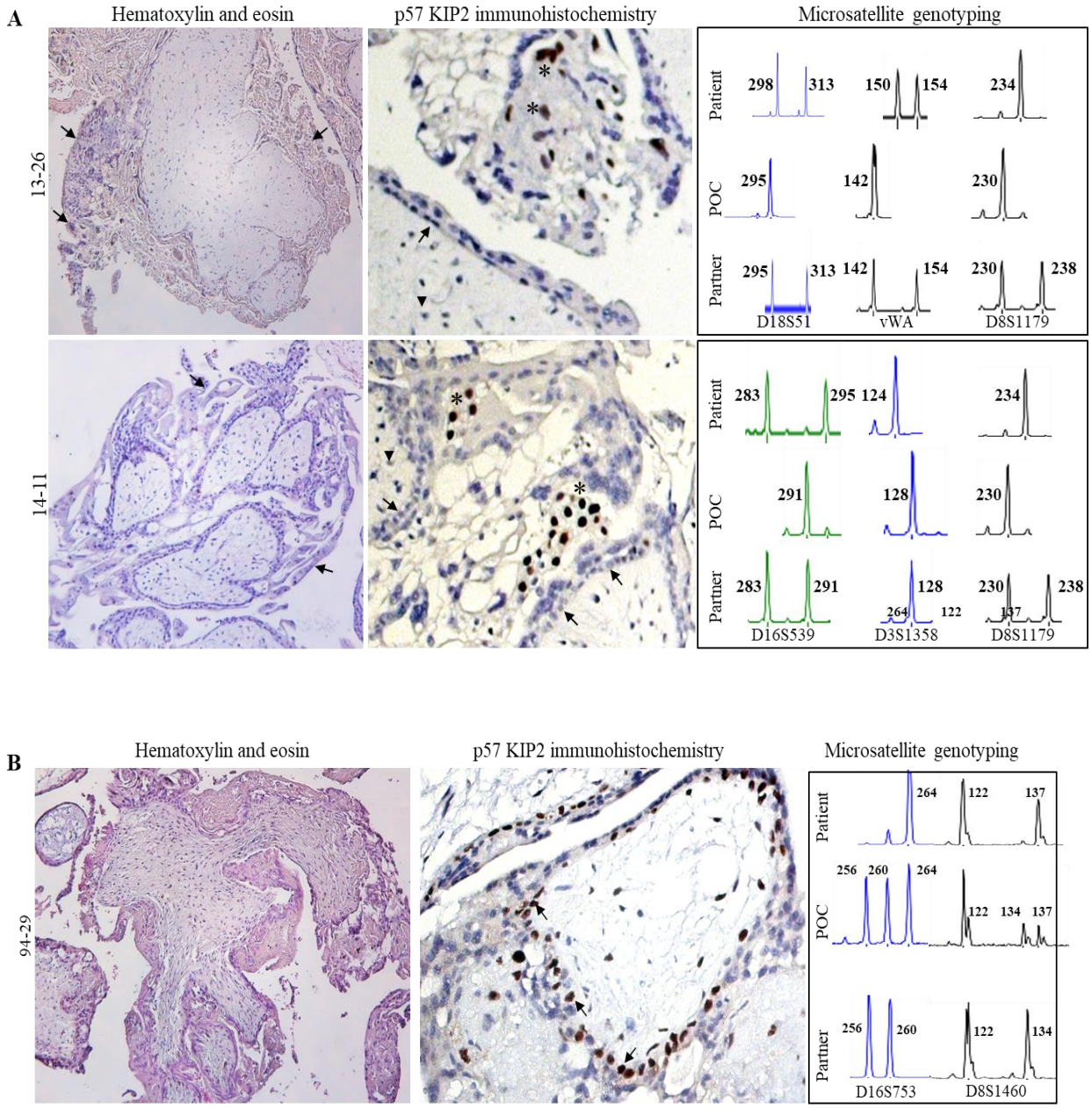


Figure 3. (A) Characterization of two HM tissues from patient 1824 with bi-allelic mutations in *MAJIN*. Left panel shows haematoxylin and eosin staining demonstrating excessive trophoblastic proliferation in the two products of conception (POC) (arrows). Middle panel shows p57KIP2

immunohistochemistry showing negative staining in the cytotrophoblast (arrows) and stroma cells (arrowheads) while the internal control, the nuclei of the extravillous trophoblast cells are positive (asterisks). Right panel shows the genotypes of the two HM shown on the left at 3 microsatellite markers revealing the presence of only one paternal allele in the molar genome at each marker. This demonstrates the androgenetic monospermic genome of the 2 HMs. (B) Comprehensive characterization of one HM tissue from the mother of the patient 1824 with homozygous protein truncating mutation in *MAJIN*. This mother has a single heterozygous mutation in *MAJIN*. Left panel shows haematoxylin and eosin staining demonstrating trophoblastic proliferation around one chorionic villus. Middle panel shows p57KIP2 immunohistochemistry showing positive staining in the cytotrophoblast (arrows). Right panel shows the HM tissue genotype at 2 microsatellite markers showing the presence of two different paternal alleles at D16S753 and three alleles at D8S1460. We note that this tissue was fixed and embedded into paraffin in 1994 (26 years ago). This is the first time, we manage to retrieve and genotype such an old tissue from the mother of a patient.

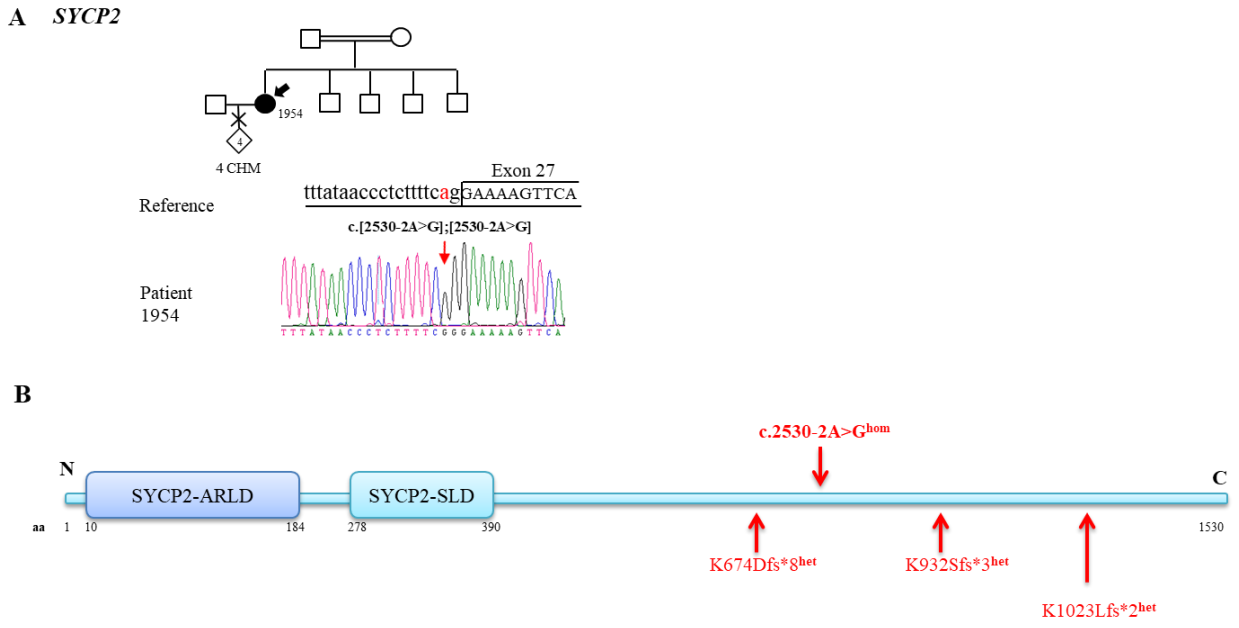


Figure 4. (A) Pedigree structure, Sanger validation, and segregation of the mutations in *SYCP2* in the family of proband 1954. (B) The protein structure with our mutation (above the protein) and previously reported mutations (below the protein) including a reciprocal translocation, 46,XY,t(20;22)(q13.3;q11.2) that leads to increased expression of *SYCP2* from derivative part of chromosome 20 due to enhancer adoption (Schilit et al., 2020). Het denotes mutations found as a single heterozygous mutation/variant and hom those found in a homozygous state.

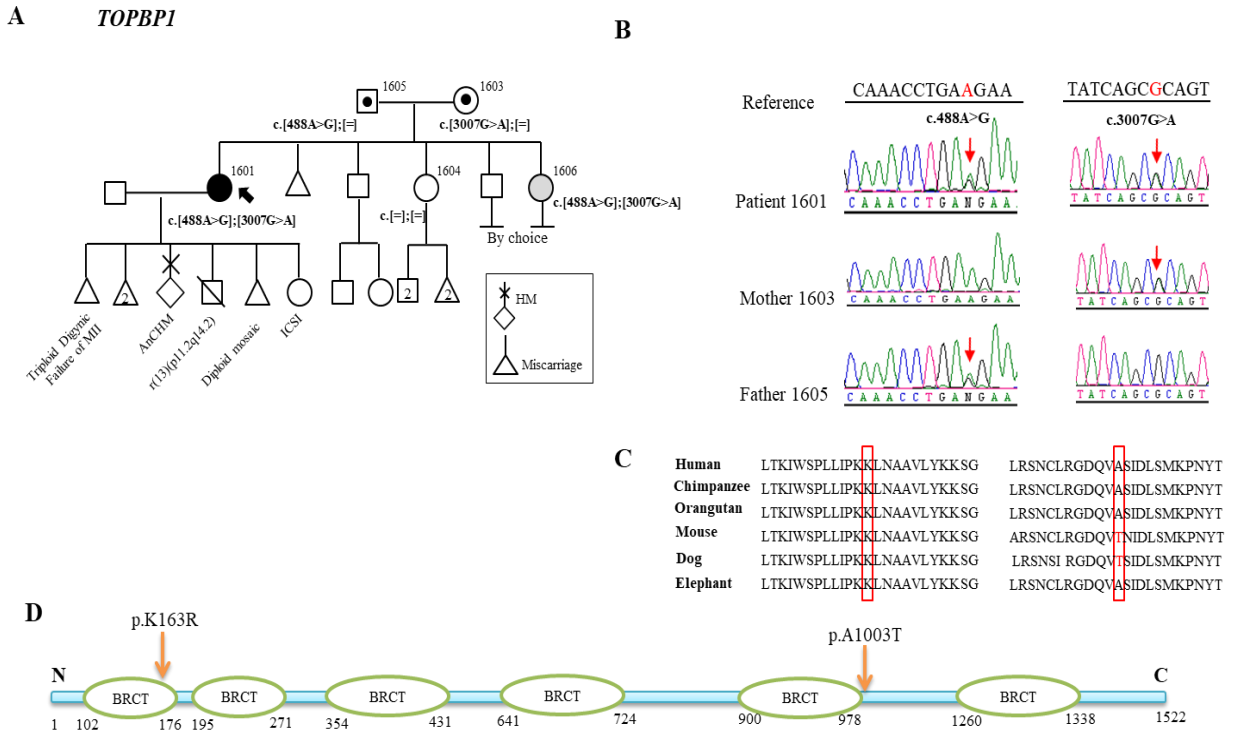


Figure 5. Pedigree structure and segregation of the two variants in *TOPBP1* in the family of proband 1601 (indicated by a black arrow). (B) Chromatograms of Sanger sequencing showing the segregation of the variants. (C) Conservation of the missense variants during evolution. (D) Structure of the TOPBP1 protein with the variants in patient 1601. AnCHM stands for androgenetic complete hydatidiform mole; the female symbol in gray indicates a woman with

unknown phenotype since she has not tried to conceive; and BRCT stands for C-terminal domain of BRCA.

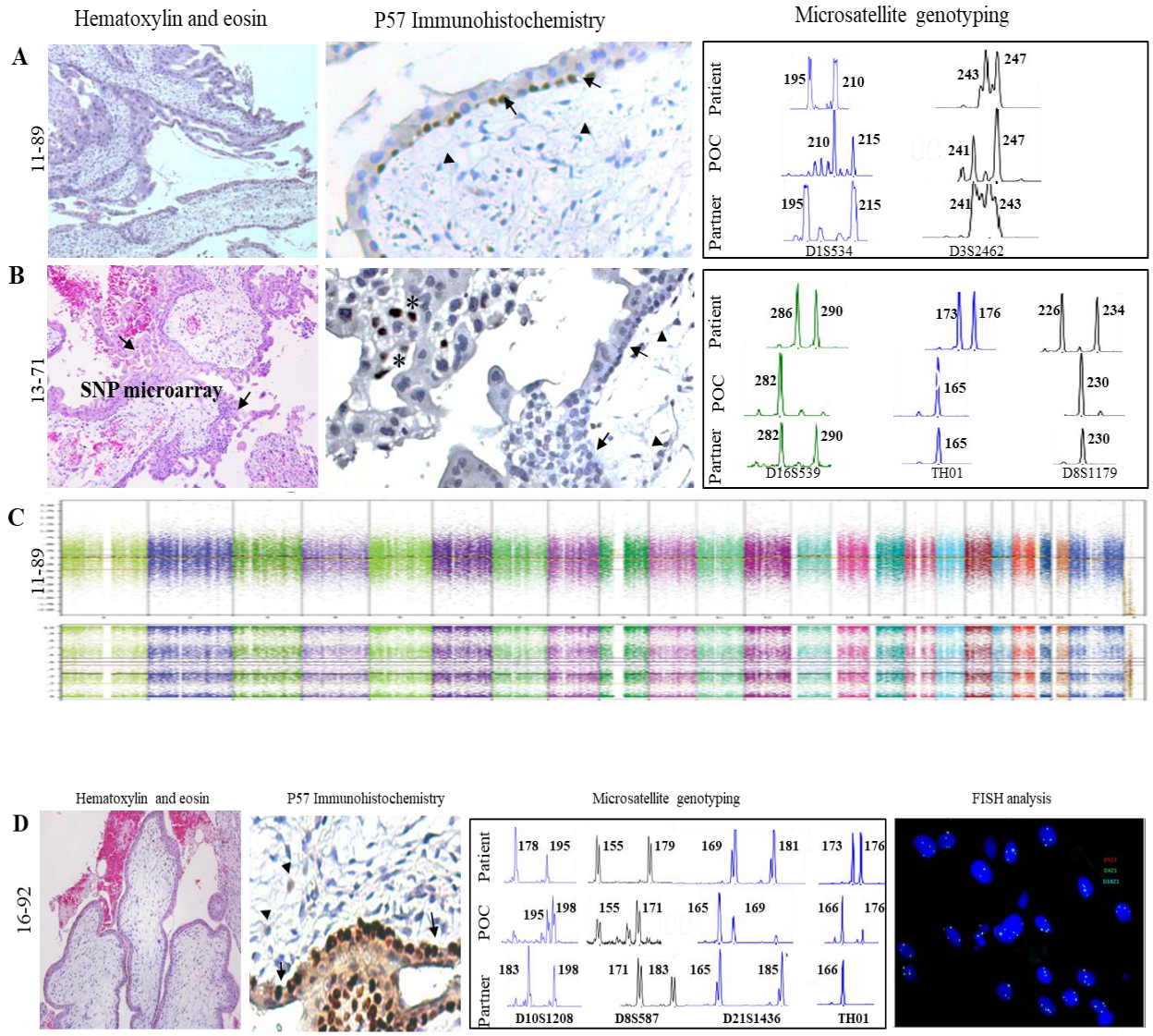


Figure 6. Characterization of three products of conception (POCs) from patient 1601 with 2 *TOPBP1* variants. (A) From left to right, top row, POC 11-89 displays some trophoblastic proliferation on some chorionic villi. Middle panel shows p57 immunohistochemistry with

positive staining in the cytotrophoblast (arrows) and negative in stroma cells (arrowheads). Microsatellite genotyping at two pericentromeric markers showed the transmission of only one of the two maternal alleles to the POC demonstrating a triploid digyny due to the failure of meiosis II. (B) POC 13-71 displays circumferential trophoblastic proliferation characteristic of complete HM. p57 immunohistochemistry showed negative staining in the cytotrophoblast (arrows) and stroma cells (arrowheads) while the internal control, the nuclei of the extravillous trophoblast cells, are positive (asterisks). Right panel shows genotypes at 3 microsatellite markers demonstrating the androgenetic monospermic genome with one paternal allele in the POC at each marker. (C) SNP microarray on the first POC 11-89 demonstrated a triploid 69,XXX genome. (D) POC 16-92 from left to right does not display trophoblastic proliferation suggesting a diagnosis of miscarriage. p57 immunohistochemistry showed strong positive staining in the nuclei of cytotrophoblastic cells (arrows) and negative staining in stroma cells (arrowheads), which was surprising. Microsatellite markers showed two doses of one paternal allele at each marker. FISH demonstrated that all cells are diploid XX. (D) FISH with centromeric probes from chromosomes X, Y and 18 confirmed the diploidy of the POC and showed the presence of two chromosomes for each probe. The conclusion of the genotype of this POC is therefore diploid mosaic with one diploid biparental and another diploid androgenetic monospermic cellular populations, both of which originating from the same zygote.

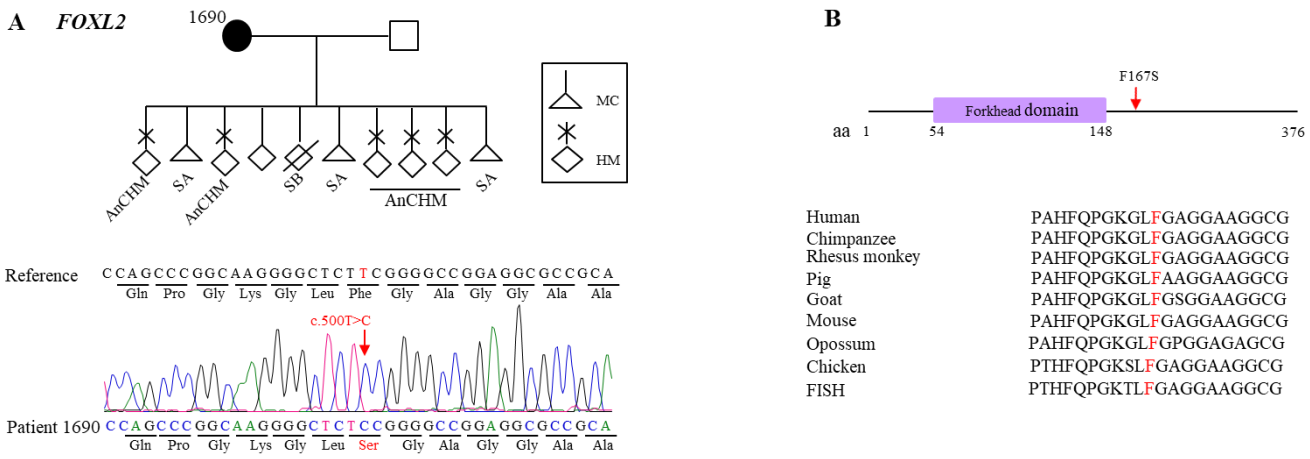
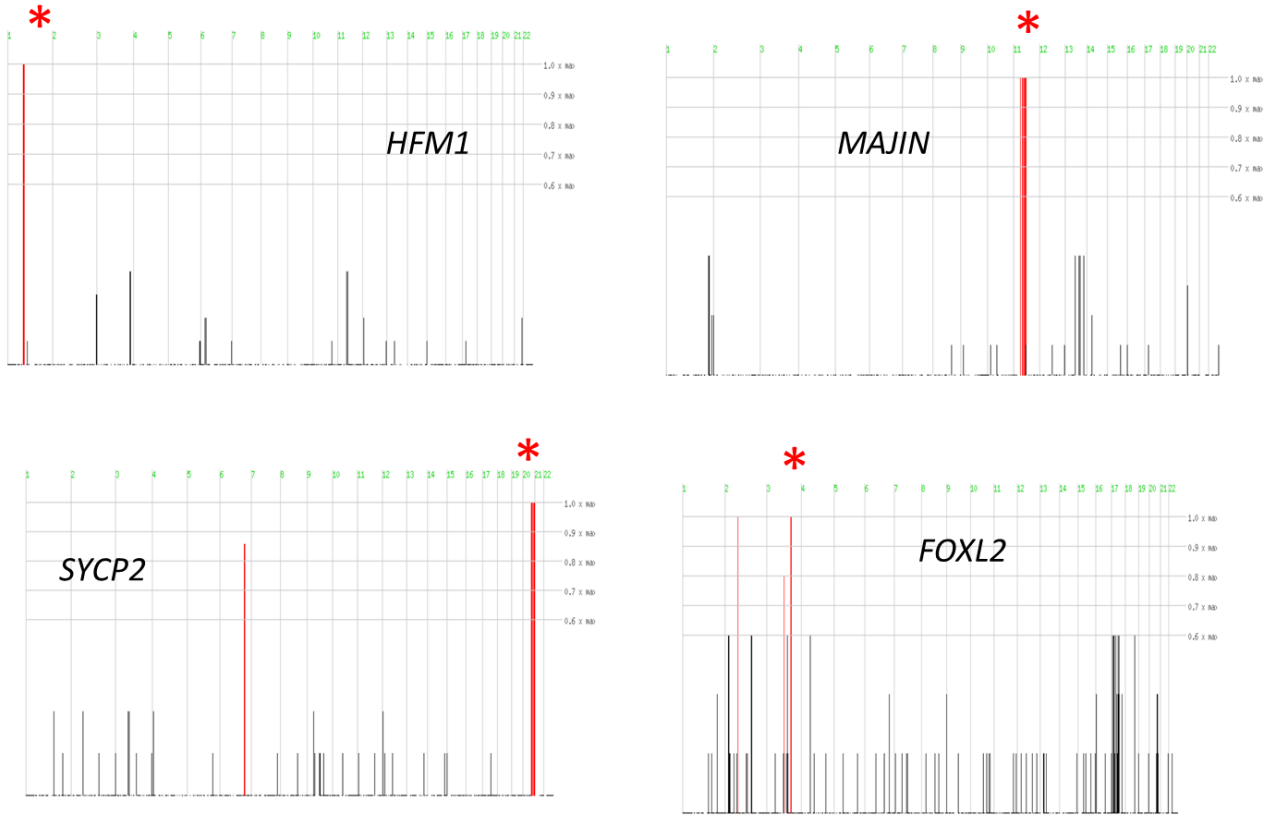
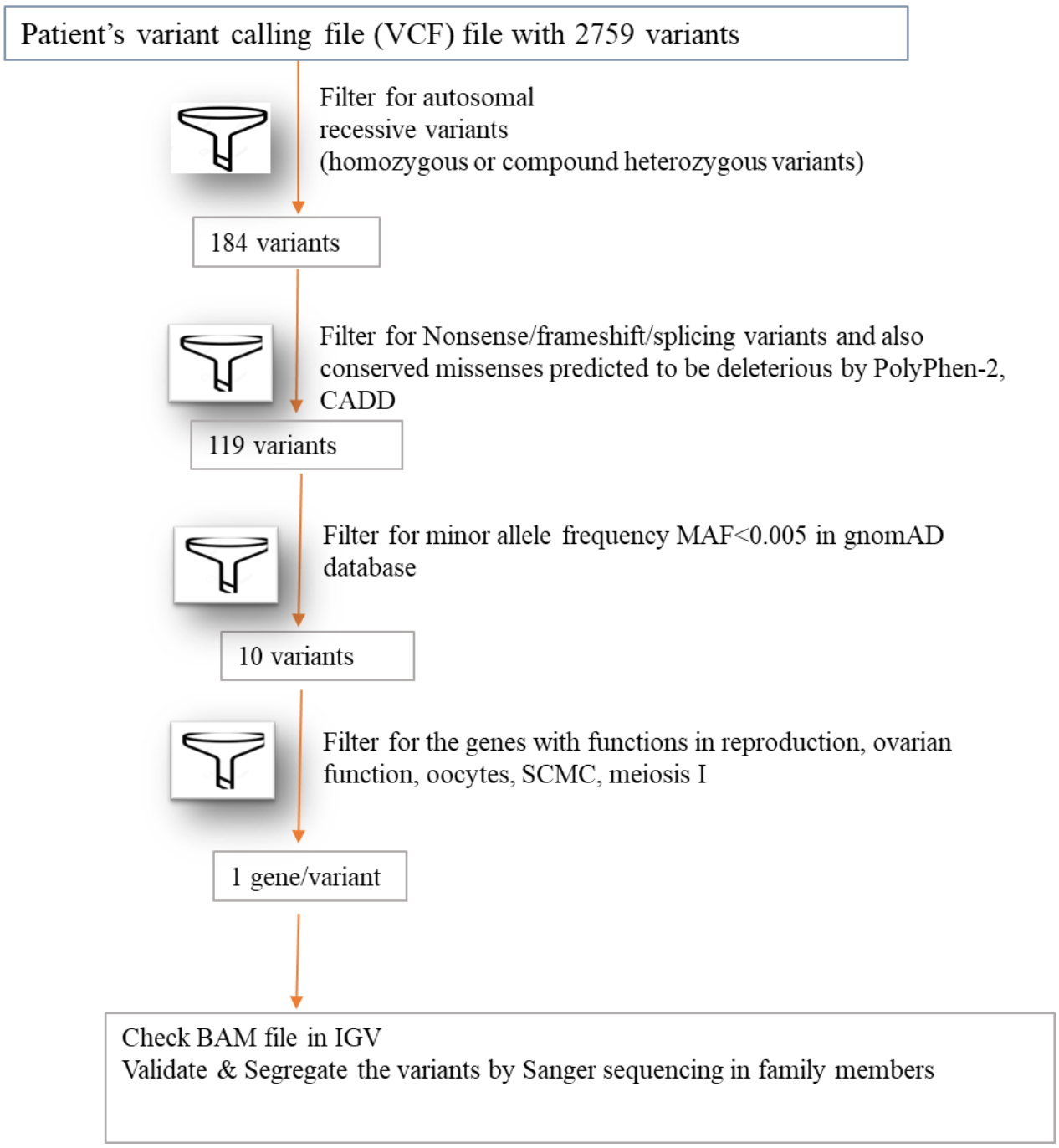


Figure 7. (A) Pedigree structure and chromatograms of Sanger sequencing of the missense variant in *FOXL2* in patient 1690. (B) Structure of the FOXL2 protein with the variants in patient 1690 and the conservation of the missense variants during evolution. AnCHM stands for androgenetic complete hydatidiform mole.

Supplementary figures
Supplementary figure 1



Supplementary figure 1: Overview of homozygosity mapping results for *HFM1*, *MAJIN*, *SYCP2* and *FOXL2* genes. The star indicates the homozygous regions harboring genes in which pathogenic variants were identified. The sizes of run of homozygosity (ROH) are: 25.07 Mb on chromosome 1for *HFM1*, 48.3 Mb on chromosome 11, for *MAJIN, 19.7* Mb on chromosome 20 for *SYCP2* and 10.47 Mb on chromosome 3for *FOXL2*.



Supplementary figure 2: The filtering pipeline for filtering the data from variant calling file (VCF) of a patient as an example.

gnomAD: Genome Aggregation Database, IGV: Integrative Genomics Viewer, CADD: Combined Annotation Dependent Depletion.

CHAPTER 5: GENERAL DISCUSSION

As a PhD student who also worked on the same disease in the master's program, I had the opportunity to investigate the pathogenesis of diploid biparental RHMs caused by recessive mutations in *NLRP7* and *KHDC3L* and diploid androgenetic RHMs caused by recessive mutations in *HFM1*, *MAJIN*, *SYCP2*, *TOPBP1* and *FOXL2*. Hydropic chorionic villi, excessive trophoblast proliferation and absence/abnormal embryonic development is the umbrella phenotype that has all the RHM formed by mutations in these seven genes under itself, despite the differences in their genotypes or other features. In this discussion, I will review my findings which are 1) Mutations in other SCMC members such as NLRP5 and PADI6 are also responsible for RHMs in very rare cases 2) Mutations in other meiotic genes such as *HFM1*, *MAJIN*, *SYCP2*, *TOPBP1* and the DNA binding protein *FOXL2* which is crucial for apoptosis in ovaries can lead to RHM as well. Identification of new genes and understanding of the consequences of defects in these genes provide us with a profound insight into the genetics of RHM and the mechanisms by which they lead to HM.

1-Genetic diagnosis of RHM patients should be started off by comprehensive and precise *NLRP7* and *KHDC3L* mutational screening

RHM are genetically heterogeneous, until now we know that bi-allelic mutations in at least 10 genes underlie its occurrence, which means all these genes must be evaluated before concluding on its possible genetic etiology. It is better to start mutation screening with *NLRP7* since it is the major gene for this condition and explains 55% of the patients. So far, 86 different mutations in *NLRP7* in 200 probands have been reported and all recorded in Infevers database. In case the patient has experienced at least 2 or more molar pregnancies and she is heterozygous for a single

NLRP7 mutation, the full search to find the second mutation should be attempted especially because NLRP7 is Alu rich and 48% of its intronic regions are Alu elements which makes this gene prone for Alu Recombination-Mediated Deletions (ARMDs) (90). I have been able to detect three large deletions (80,81,90,92) (approximately 11 kb, 10 kb and 14 kb respectively) in NLRP7 which were the first regulatory mutations that remove the entire *NLRP7* promotor (spans from 499-bp upstream of exon 1 to 63-bp downstream of exon 1), and all were caused by Alu elements. I was able to detect these deletions using either digital droplet PCR or long range PCR, where Cytoscan HD microarray could not help us due to its low SNP content in the genomic structure of NLRP*7*.

Regarding the study on *NLRP7* mutational screening, I screened the DNA for 37 patients including seven Mexican patients which I analyzed the haplotype (from rs775886 to rs269933 spanning 18,296 bp, covered by Sanger sequencing) carrying the founder mutation, which appears to be specific for this population. Therefore, for this specific population, it is recommended to begin the mutation analysis by sequencing exon 6 of *NLRP7*. In case the patient is negative for the common mutation, sequencing of the whole NLRP7 can be continued. The highest number of patients with L750V was observed in the center/south of the country where in Mexican population the Native American ancestry is highest (108), which suggests this strong founder effect may have been inherited from the Native American population that needs to be studied in the future.

In this study we also reported the two live births from patients with less sever *NLRP7* mutations, from spontaneous conceptions that led to healthy children. This shows the importance of genetic counselling for patients with bi-allelic mutations in *NLRP7* about their future decisions and options.

In case the patient is negative for mutational screening for *NLRP7*, the next gene that needs to be checked is *KHDC3L* which explains about 5 % of RHM cases (18). In *KHDC3L*, to date seven mutations have been reported.

Of note, almost all HM due to mutations in *NLRP7* and *KHDC3L* genes that were genotyped were found diploid biparental with the exception of two HM caused by *NLRP7* mutations that were found to be triploid. These *NLRP7* caused triploidies shed light on first, bi-allelic *NLRP7* variants can cause another meiotic abnormality, though rarely, and second, *NLRP7* defects in the oocytes are the main contributor to the molar phenotype, independent of the zygotic genome (42).

2- *NLRP5* and *PADI6* are also responsible for RHM but in a minority of cases

We reported the first patient with homozygous missense mutation in *NLRP5* and two patients with novel missenses, which explain approximately 0.5% and 1% of RHM cases, respectively (2,80). From now on, these two genes need to be checked in case the patient is negative for mutations in *NLRP7* and *KHDC3L*.

NLRP5 or *MATER* (Maternal antigen that embryos require), the first maternal-effect gene that was found in mice and its absence leads to female infertility in mice due to early embryonic arrest at the two-cell stage (59). In humans, mutations in *NLRP5* have been associated with primary infertility in f (86,127–131), recurrent miscarriages as well as children with imprinting defects such as BWS, SRS, MLID (67,84). By finding the first patient with bi-allelic mutations in *NLRP5* who had experienced 3 HMs after a couple of years of primary infertility, we expanded the phenotypic spectrum of *NLRP5* mutations in humans (80).

In *PADI6*, our group was also the first to associate bi-allelic mutations in PADI6 mutation with RHMs (2,80). Peptidyl Arginine Deiminase 6, converts arginine residues to citrulline and is required for the formation of the oocyte lattices that are believed to work as ribosomal storage for early embryo (68). Both mouse and human null *PADI6*, show early embryonic arrest and infertility by impairing ZGA (79,115). In the first study, that we reported the first patient with one HM and recurrent miscarriage, I investigated the localization of PADI6 relative to NLRP7 in human oocytes and early cleavage embryos because of three reasons: first in mice, it was already known that PADI6 is an SCMC member, second it plays a causal role in early embryonic development in humans, and third the missense mutation in our patient with recurrent miscarriage and one HM. This immunofluorescence analysis demonstrated that PADI6 in human oocytes has a similar localization to that observed in mice and is abundant in the subcortical region and co-localizes with NLRP7 (Pearson coefficient = 0.81) (2). In the second study, we reported the second patient with bi-allelic mutation in *PADI6* who had experienced seven pregnancy losses, four HMs including two with fetuses that have features of BWS, and SRS (80). Alongside with this observation, two groups had found four women with bi-allelic *PADI6* mutations who had six children with BWS, and MLID at multiple loci (68,94). In one study, there were three families, with 4 BWS children. In the second study, the patient who had bi-allelic mutation in *PADI6*, had experienced three pregnancy losses and 2 children born with some clinical features of BWS, all from the same father. In both studies it was pointed out that there are overlapping features of both BWS (placental mesenchymal dysplasia and macroglossia) and SRS (small for gestational age (SGA) and protruding forehead) which was similar to our findings in the fetuses of our patient, which all show that *PADI6* variants may result in a wide range of DNA methylation alterations and clinical phenotypes in the offspring. Altogether, these

data confirms that maternal-effect variants affecting the components of the oocyte SCMC, such as PADI6 and NLRP5 expand the phenotypic spectrum of reproductive outcome to RHMs in the case of NLRP5 and RHMs, miscarriage and live births with imprinting disturbances at multiple loci in *PADI6* case. Thus, in human, after NLRP2, NLRP7 and KHDC3L, NLRP5 and PADI6 represent the fourth and fifth SCMC members in which maternal-effect variants affect the reproductive outcome with a spectrum of abnormalities thatincludes female infertility, hydatidiform moles, miscarriages and live births with multi-locus imprinting disturbance (Figure 5.1).

A precise genetic and reproductive counseling should be offered to the patients with pathogenic maternal-effect variants in all SCMC members, since these patients are at high risks for further reproductive loss. Furthermore, as it was mentioned in the cases with MLID children, due to the mosaic distribution and heterogeneity of imprinting disturbances, ovum donation is a feasible option.

Of note, because our lab focuses mainly on NLRP7 and KHDC3L, two SCMC members, so far in patients with bi-allelic *NLRP7* variants, 12 patients achieved a term pregnancy with their own oocytes despite two experienced early neonatal death, and one exhibited intrauterine growth restriction. Ovum donation in the same patients, has enabled at least 11 successful live births (42).

3- The genetics of recurrent androgenetic hydatidiform moles has started to be unraveled

In 2018, our lab identified three meiotic genes, meiosis inhibitor 1 (*MEI1*), type 2 DNA topoisomerase VI subunit B-like (*TOP6BL*), and meiotic recombination protein 114 (*REC114*) which are responsible for recurrent androgenetic monospermic HM. These patients had bi-allelic

mutations and their reproductive history in addition to HM included miscarriages, female subfertility and male infertility. In this study by using *Mei1*$^{-/-}$ mouse model, it was found that 8% of mutant oocytes lose their chromosomes and spindles into the first polar body (PB) and were therefore "empty". Additionally, it was found that 5% of the zygotes derived from *Mei1*$^{-/-}$ oocytes had lost their spindles with all the maternal chromosomes at the poles into the first PB and were androgenetic. Thus, our group demonstrated for the first time the occurrence of androgenetic zygotes in a mouse model and a plausible mechanism for the genesis of androgenetic moles (3). These three meiotic genes are involved in DSB formation during prophase of meiosis 1.

Following the path of identification of novel genes for recurrent HMs, during my project, we identified five new candidate genes for recurrent androgenetic HMs, helicase for meiosis I (*HFM1*), membrane anchored junction protein (*MAJIN*), synaptonemal complex protein 2 (*SYCP2*), topoisomerase II binding protein 1 (*TOPBP1*), in five patients including two sisters and forkhead box 2 (*FOXL2*) in another patient. Four out of five genes are involved during prophase pf meiosis 1.

4- Is meiosis prophase 1 failure the cause of recurrent androgenetic hydatidiform moles?

In sexually reproducing organisms, the chromosome content of diploid germ cells must be halved during gamete formation. Halving the genetic complement occurs through two successive rounds of cell divisions without intervening DNA replication. During the first division (the reductional division), homologous chromosomes segregate from each other, whereas during the second division, sister chromatids segregate. One hallmark of meiosis is recombination which

plays an important role in increasing genetic diversity during inheritance and pairing/segregation of homologs (132).

Recombination initiates by programmed double-strand breaks (DSBs) which mediates the coalignment of homologs with a distance of ~400 nm in "leptotene"(133). Following coalignment, synapsis happens through the installation of a proteinaceous structure, synaptonemal complex (SC), between the homolog axes with a distance of ~100 nm in "zygotene". The presence of complete SC defines "pachytene." Crossovers (COs) or reciprocal recombination events between homologous chromosomes appear at the end of pachytene, and after the SC disassembles and homologs separate along their lengths, except at the sites of COs (visualized in the cell as chiasmata), in "diplotene"(134).

Meiotic recombination is initiated by the formation of DNA double-strand breaks (DSBs). Meiotic DSBs are catalyzed by the evolutionarily conserved topoisomerase-like protein Spo11, which shares similarity with the catalytic subunit of type II DNA topoisomerases. The number of Spo11-induced DSBs per genome is regulated on a species basis and tend to be higher in organisms with longer chromosomes (in mouse, ~200–300 DSBs per meiotic cell). DNA ends are then resected 5′ to 3′ by Exo1 exonuclease to expose 3′ single-stranded tails. A 3′ ssDNA tail containing RecA family of strand exchange proteins (Dmc1, Rad51) invades a homologous duplex DNA and initiates repair synthesis. DSBs are repaired by homologous recombination using the homologous chromosome as a template, leading to a gene conversion either without CO (NCO) or with CO.

The three meiotic genes that were found by Nguyen et al, are involved in DSB formation in prophase 1. In terms of the meiotic genes' functions from my project, MAJIN attaches telomere

DNA to the nucleus inner membrane and leads to chromosome movement and consequently homologous pairing and synapsis during meiotic prophase I (118,135). TOPBP1 is involved in double-stranded DNA break formation during homologous recombination repair (136). HFM1 is required for completion of synapsis and maximum number of CO events between homologous chromosomes. (117). SYCP2 codes a component of the axial/lateral elements of synaptonemal complexes (SC) required for normal meiotic chromosome synapsis during oocyte and spermatocyte development (137). So based on all these findings, it seems recurrent androgenetic hydatidiform moles are due to meiosis 1 failure.

5- Identification of candidate genes responsible for recurrent pregnancy loss has its own challenges

The search for genes responsible for recurrent pregnancy loss presents unique challenges given the complexity of the process of oogenesis, with so many genes involved in this process (138). Given this complexity, it is not surprising that the majority of recurrent pregnancy loss associated variants or specifically recurrent hydatidiform moles identified to date have been found in a very small percentage of the patients (Figure 5.2). The assembly of large cohorts of patients with RHMs, thorough genotyping, and the development of genome analysis tools with deep annotation of reproductive data and efficient use of animal models are critical for continued gene discovery.

Figures

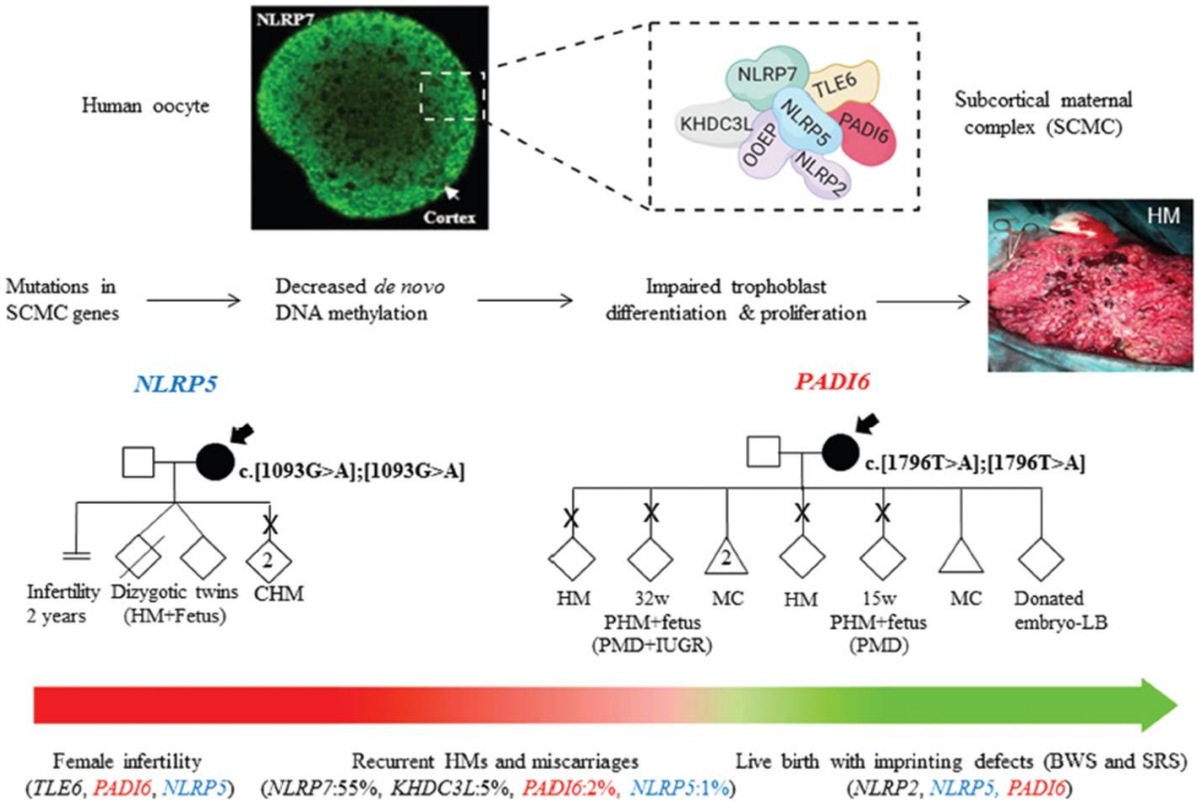


Figure 5.1. Recapitulation of the data on subcortical maternal complex membershowed a clear continuous spectrum of conditions caused by mutations in SCMC genes that originates in the oocyte, goes beyond infertility and early pregnancy losses (e.g HM, miscarriages) and includes live births with imprinting disorders.

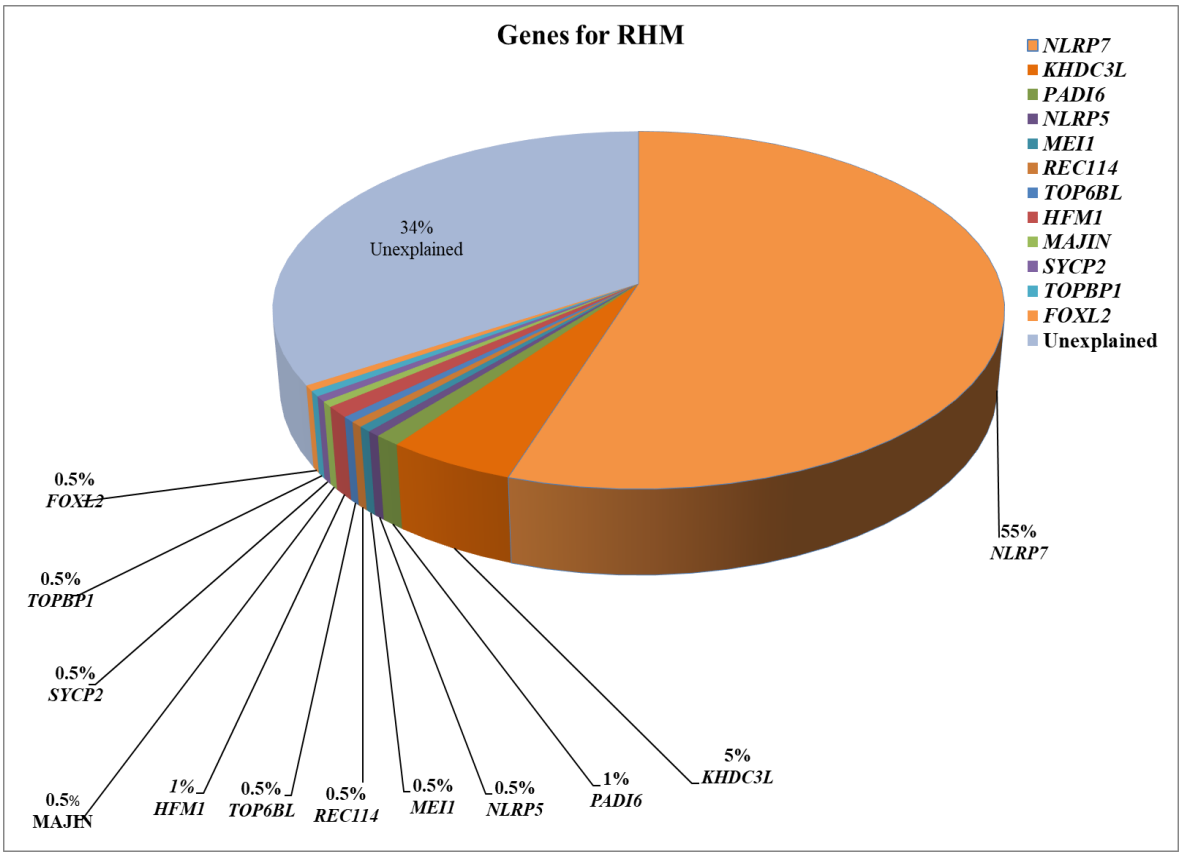


Figure 5.2. Responsible genes for RHM**.** Percentages of patients with RHM and bi-allelic pathogenic variants in the 12 known genes.

CHAPTER 6

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APPENDIX (ETHICS AND COPYRIGHT PERMISSION)

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CERTIFICATION OF ETHICAL ACCEPTABILITY FOR RESEARCH INVOLVING HUMAN SUBJECTS

The Faculty of Medicine Institutional Review Board consisting of:

ARTHUR CANDIB, MEd	PATRICIA DOBKIN, PhD
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has examined the research project **A01-M07-03A** entitled **"Identification of a Gene Responsible for Repeated Hydraditiform Moles"**

as proposed by: Rima Slim (Applicant) to _____ (Granting Agency, if any)

and consider the experimental procedures to be acceptable on ethical grounds for research involving human subjects.

January 24, 2003 (Date) — Chair, IRB — Dean of Faculty

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FACULTY: MEDICINE

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