Genetic susceptibility to recurrent miscarriages: identification of patients with a unique and defined mechanism

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Abstract

Miscarriages affect 15% of clinically recognized pregnancies. The recurrent form of miscarriages (RM) is defined by the occurrence of at least two pregnancy losses and affects 1% to 5% of couples trying to conceive. RM are clinically and genetically highly heterogeneous. One of the many factors that has hampered the identification of causative or susceptibility genes for RM lies in the high genetic heterogeneity of this clinical entity. In an attempt to categorize the entity of RM and divide the patients into more categories according to the mechanisms leading to their RM, we first used flow cytometry to assess the ploidy of 96 products of conception (POCs) from 54 patients with at least three RM (≥ 3 miscarriages). We identified six triploid POCs (6%), of which three are from unrelated patients and three are from the same patient. We then used fluorescent in situ hybridization to confirm the triploidies and fluorescent microsatellite genotyping with distal and pericentromeric markers to determine their parental origin and the mechanisms leading to their formation. We found that all six triploidies are digynic. Among the three triploidies from unrelated patients, one was due to failure in meiosis I and two due to failure in meiosis II. The three triploidies from the same patient were found to be due to failure in meiosis II. Interestingly, three out of the four patients with triploidies had polycystic ovaries. This high rate of maternal triploidies among patients with RM and polycystic ovaries suggests a possible association between the two entities. Identifying such patients with a specific abnormality and mechanism of RM will facilitate the identification of causative genes for this condition and will allow for the provision of better genetic counselling and appropriate ART services for the patients.

Résumé

Les fausses couches affectent 15% des grossesses cliniquement reconnues. Les fausses couches à répétition sont définies par au moins deux pertes de grossesse et affecte 1% à 5 % des couples essayant de concevoir. Les fausses couches à répétition sont cliniquement et génétiquement très hétérogènes. L'un des nombreux facteurs qui a entravé l'identification des gènes responsables des fausses couches à répétition réside dans l'hétérogénéité génétique de cette entité clinique. Dans une tentative de classer l'entité des fausses couches à répétition et de diviser les patientes en plusieurs catégories selon les mécanismes conduisant à leurs fausses couches à répétition, nous avons utilisé la cytométrie en flux pour évaluer la ploïdie de 96 produits de conception conservés dans des blocs de paraffine de 54 patientes avec au moins trois fausses couches (≥ 3 fausses couches). Nous avons identifié six conceptions triploïdes (6%), dont trois sont chez la même patiente et trois autres sont chez des patientes différentes non-apparentées. Ensuite, nous avons utilisé l'hybridation in situ observée en fluorescence pour confirmer les triploïdies et le génotypage par des marqueurs microsatellites visualisés en fluorescence (marqueurs distaux et péricentromériques) afin de déterminer leur origine parentale et investiguer les mécanismes conduisant à leur formation. Nous avons trouvé que les six triploïdies sont digyniques (d'origine maternelle). Parmi les trois triploïdies présentent chez des patientes différentes, une est due à un échec de la méiose I et deux à un échec de la méiose II. Par contre, les trois triploïdies présentent chez la même patiente sont dues à un échec de la méiose II. Fait intéressant, trois des quatre patientes avec triploïdies avaient été diagnostiquées avec des ovaires polykystiques. Ce taux élevé de triploïdies maternelles chez les patientes qui présentent des fausses couches à répétition et des ovaires polykystiques suggère une association possible entre les deux. L'identification de ces patientes avec une anomalie et un mécanisme spécifique de fausses couches à répétition facilitera l'identification des gènes responsables de leurs fausses couches et permettra d'assurer un meilleur conseil génétique et un suivi approprié de services de technologie de reproduction assistée à ces patientes.

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Abbreviations

2H OGTT	2 hour oral glucose tolerance test
ANA	Antinuclear antibodies
APCR	Activated protein C resistance
ART	Assisted reproductive technology
AT1II	Antithrombin 1II
bp	Base pairs
BO	Blighted ovum
BSA	Bovine serum albumin
CADD	Combined annotation dependent deletion
CBC	Complete blood count
CENPH	Centromere protein H
CI	Confidence interval
CMV	Cytomegalovirus
Coag	Coagulation
CP	Chemical pregnancy
CRRD	Centre for Research in Reproduction and Development
Ctl	Control
CV	Chorionic villi
DAPI	4,6-diamidino-2-phenylindol-2-HCl
DHEAS	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EP	Ectopic pregnancy
eTOP	Elective termination of pregnancy
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
GBS	Group B Streptococcus
GV	Germinal vesicle
GWAS	Genome wide association study
FSH	Follicle stimulating hormone
FISH	Fluorescent in situ hybridization
G6PD	Glucose-6-phosphate dehydrogenase
GC	Neisseria Gonorrhoeae culture
Gen'l	Genital tract culture
GV	Germinal vesicle
H6PD	Hexose-6-phosphate dehydrogenase/Glucose 1-dehydrogenase
H&E	Hematoxylin and eosin
hCG	Human chorionic gonadotropin
HCl	Hydrogen chloride

Hep B	Hepatitis B
Hep C	Hepatitis C
Hgb	Hemoglobin
HIV	Human immunodeficiency virus
HM	Hydatidiform mole
HOS	Hypo-osmotic swelling
HSG	Hysterosalpingogram
Ins	Insulin
IUFD	Intra-uterine fetal death
IUGR	Intra-uterine growth restriction
IUI	Intra-uterine insemination
IVF	*In vitro* fertilization
Kb	Kilo bases
LAC	Lupus anticoagulant
LB	Live birth
LH	Luteinizing hormone
M	Molar; mole per liter
MAF	Minor allele frequency
Mb	Mega bases
MI	Meiosis I
MII	Meiosis II
ml	Milliliter
mmol	Millimole
mRNA	Messenger RNA
MTHFR	Methylene tetrahydrofolate reductase
MUHC	McGill University Health Center
NA	Not available
NaCl	Sodium chloride
NR	Non-reduction
Parvo	Parvovirus
PBMC	Peripheral blood mononuclear cells
PBS-1x	Phosphate-buffered saline
PCO	Polycystic ovaries
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGD	Prenatal genetic diagnosis
PGS	Prenatal genetic screening
PI	Propidium Iodide
Plat	Platelet
POC	Product of conception
POR	Prevalence odds ratio

PR	Pregnant
Prot C	Protein C
Prot S	Protein S
PSRC1	Proline and serine rich coiled-coil 1
RF	Rheumatoid factor
RM	Recurrent miscarriage
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SA	Spontaneous abortion
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
TOP	Termination of pregnancy
Toxo	Toxoplasmosis
TPO	Thyroid peroxidase
TSH	Thyroid-stimulating hormone
UI	Uninformative
µl	Microliter
US ab	Ultrasound abnormality
VDRL	Venereal disease research lab (test for syphilis)
w	Weeks

Chapter 1: Introduction - Recurrent Miscarriages

Introduction

First trimester reproductive loss may manifest in the form of a chemical pregnancy, an ectopic pregnancy, a blighted ovum, a miscarriage, or a hydatidiform mole (HM). A chemical pregnancy is defined by a positive pregnancy test that detects the presence of the pregnancy hormone hCG in blood or urine. hCG is produced by the syncytiotrophoblast, the external layer of trophoblastic cells that surround chorionic villi, and is detected in maternal blood seven days after fertilization. A chemical pregnancy is therefore a very early miscarriage (< 5 weeks of gestation), in which the blastocyst implant, the trophoblast differentiates into cytotrophoblast and syncytiotrophoblast, but the pregnancy fails to develop soon after implantation. An ectopic pregnancy is defined by the implantation of the blastocyst outside the uterus, usually in one of the Fallopian tubes. A blighted ovum (whose name is wrong and inaccurate) is an early arrested pregnancy in which there is a visible gestational sac by ultrasound but no evidence of the presence of an embryonic pole or fetal heart activity. HM is an abnormal human pregnancy characterized by absence of, or abnormal, embryonic development, excessive trophoblastic proliferation, and hydropic degeneration of placental villi.

A miscarriage, also known as a spontaneous abortion, is the most common form of fetal loss and is defined by the spontaneous arrest of the pregnancy before 24 weeks of gestation (according to some researchers[1]) or 20 weeks of gestation (according to the American Society for Reproductive Medicine[2]). Recurrent

miscarriage (RM) is defined by two or more miscarriages and affects 1% to 5% of couples trying to conceive.[3] Clinicians divide patients with RM into two subgroups, primary and secondary RM. A primary RM refers to patients who have never had a live birth before their first miscarriage and secondary RM refers to patients who have had at least one live birth before their first miscarriage. Some studies have suggested that these two entities are separate and may have different aetiologies. Primary RM is believed to be more severe, and more likely to be caused by an underlying genetic problem that leads to repeated occurrence of the same mechanism. It has been shown that there is an increased risk for adverse obstetric outcomes, such as preterm delivery, fetal growth restriction, and gestational diabetes mellitus, in patients with primary RM.[4] Immunological factors are thought to play a greater role in secondary RM, especially when the first live birth is a boy.[5, 6] It is proposed that immunization against male-specific minor histocompatibility antigens, which are only present in males, in the first ongoing pregnancy could account for this.[6]

Because of the multifactorial causes of sporadic miscarriages and the fact that a minority of the patients have RM, comprehensive clinical evaluations of the underlying causes of miscarriages is usually restricted to patients with at least two, and most commonly three, miscarriages. For instance, in the Canadian public health system as well as in many western countries, karyotype of the parental blood and/or the miscarriages is performed starting from the third miscarriage.

Clinical presentation, diagnosis of miscarriages, and management

Patients with miscarriages usually present with vaginal spotting or bleeding, abdominal pain, and/or passage of tissues. After the tissue passes, the vaginal bleeding and abdominal pain subsides.[7] Diagnosis is performed by human chorionic gonadotropin (hCG) testing and ultrasound - serial quantitative hCG blood tests are done, usually two to three days apart. In early pregnancy, the hCG level usually doubles every two to three days; if the hCG doubling time is slower or if the level decreases over time, it indicates non-growing trophoblastic cells and consequently a non-viable pregnancy or a miscarriage or an ectopic pregnancy. An ultrasound can reveal, for example, the absence of an embryo, or embryonic demise, defined by the presence of an embryonic fetal pole without cardiac activity.[8] Expectant management, which is becoming increasingly popular, is when the woman opts to wait for the pregnancy to resolve spontaneously. Such patients are advised to wait two weeks for the miscarriage to resolve spontaneously, and only if there are no signs of infection.[8] Under medical management, the drug most commonly used to induce an abortion is a prostaglandin analogue, misoprostol, also known as Cytotec. This drug works by selectively binding to specific prostanoid receptors and stimulating contractions, which push the products of conception out. More traditionally, surgical curettage was performed to evacuate the uterus.[9]

Histopathological diagnosis

In Canada and most western countries, histopathological examination of products of conception from miscarriages is systematically performed on all arrested pregnancies regardless whether they are sporadic or recurrent. However, the

histopathological evaluation adds little to the identification of the causes of the miscarriages, except for rare cases of chronic intervillositis, villitis, massive perivillous fibrin deposition/maternal floor infarction, and plasma cell deciduitis, which are important because they are associated with an increased recurrence risk.[8]

Clinical and laboratory evaluations of patients with recurrent miscarriages

In most western countries, comprehensive evaluations of patients with RM starts after three pregnancy losses and includes various organs and systems to investigate the presence of male or female aetiologies at the origin of the RM.

Paternal factors

Infertility affects about 10-15% of males in their prime reproductive years.[10] Though it is multifactorial in nature, infertility due to paternal factors is not yet completely understood, and half of the cases remain unexplained.[11] The main paternal factors that are tested include sperm quality and quantity, and chromosomal abnormalities. Semen parameters are evaluated for the following: volume, sperm count, total and progressive motility, vitality, and morphology. Assessment can reveal several semen abnormalities, including: aspermia, azoospermia, hypo/hyperspermia, oligozoospermia, asthenozoospermia, teratozoospermia, necrozoospermia, and leucospermia, as shown in table 1.1.

Table 1.1. Male factor abnormalities detected by semen analysis

Abnormality	Definition
Aspermia	Absence of semen
Azoospermia	Absence of sperm
Hypospermia	Low semen volume
Hyperspermia	High semen volume
Oligozoospermia	Very low sperm count
Asthenozoospermia	Poor sperm motility
Teratozoospermia	Morphologically defective sperm
Necrozoospermia	Presence of dead sperm in the ejaculate
Leucospermia	High levels of white blood cells in the semen

Nonobstructive azoospermia is one of the leading causes of male infertility and affects 5-10% of infertile men. It is a condition characterized by the absence of sperm in ejaculates because of spermatogenic failure.[12] Special attention is also given to varicocele, which is an enlargement of veins in the scrotum that affects sperm production and quality, and is another leading cause of male infertility (found in about 40% of the infertile male population).[13] Current research supports the role of oxidative stress in the pathophysiology of varicocele; as such, assessment of sperm DNA fragmentation is a means that provides information about the extent of oxidative stress.[13]

Another sperm quality test that is under investigation is the sperm hypo-osmotic swelling (HOS) test score. Buckett et al. compared the sperm HOS test scores of 20 men of couples with RM and 20 male donors, and found a significantly positive correlation between RM and lower HOS scores.[14] These findings were replicated in another recent study by Check et al., who found that low HOS scores almost invariably negatively affect embryo implantation, and concluded that the HOS abnormality may be the most reliable semen abnormality predicting failure to conceive.[15] Unfortunately, the HOS test is still only rarely used by infertility specialist.[15] Gopalkrishnan et al. found an association between poor sperm quality and RM. In addition, the authors of this study used specific markers to assess the quality of the sperm and found an association between the increased number of sperms' nuclear vacuoles or abnormal chromatin condensation and increased RM.[16]

It is not surprising that sperm quality can have a major impact on early embryonic development. Firstly, the sperm provides the centrosome in the first mitotic division after fertilization.[17] Secondly, it does provide half of the genome

after all, and it has been shown that imprinted, paternally expressed genes play a crucial role in trophoblastic differentiation, invasion, and proliferation.[18] Animal studies have shown that androgenotes, embryos created with two male gametes, have well developed placentas, with very little embryonic development or even no embryo.[19] This emphasizes the importance of the male genome for placental development.

Chromosomal abnormalities that can be revealed from karyotyping the father's blood can be due to an abnormal total number of chromosomes or an abnormal structure of individual chromosomes. An abnormal number of chromosomes usually leads to a miscarriage. For instance, men with an extra chromosome 21 (Down's syndrome) and those with an extra X chromosome (Kleinfelter's syndrome) do survive but most have significantly reduced fertility or sterility and have high miscarriage rates of 50% or more.[20] A translocation is a structural chromosomal abnormality that results in a different arrangement of the appropriate number of chromosomes, meaning that the genetic material is all present but not always in the right places. In such cases, it is possible for these individuals to create unbalanced gametes, and the resulting conception typically aborts. Individuals with balanced translocations do not have any clinical or physical findings, but are at higher risk of a miscarriage because of abnormal pairing between homologous chromosomes in the gametes. Balanced translocations include reciprocal or Robertsonian translocations; the prevalence of Robertsonian translocations in couples with RM is about 8%.[21]

Another possible cause of paternal chromosomal abnormalities is sperm DNA damage. The extent of sperm DNA damage in subfertile men (with

oligozoospermia and asthenozoospermia) was shown to be significantly higher than in healthy men.[22] These subfertile men also have an elevated risk of sperm chromosomal abnormalities, suggesting that DNA damage may be involved in the abnormal segregation of chromosomes and consequently to aneuploidies in the conceptions.[22]

Nowadays, it is possible to look at the karyotype of single sperm – a study by Carrell et al. found that the average sperm aneuploidy rate in men of unexplained RM couples was significantly higher than that of fertile controls ($P<0.001$).[23]

Several studies have shown the existence of other paternal factors that are not yet commonly assessed. These include certain gene mutations and paternal age.

Gene mutations in a number of genes have been associated with miscarriage. The common ones include the HLA-G polymorphisms, thrombophilia mutations, and microdeletion of the Y chromosome. HLA-G is a nonclassical human leukocyte antigen expressed primarily in fetal tissues at the maternal-fetal interface. Its expression plays a role in establishing and maintaining the fetal-placental unit in early pregnancy. Aldrich et al. found that the HLA-G 0104 or HLA-G 0105N carrier in either partner was associated with RM. Furthermore, they found that the carrier rate of either alleles was higher in couples with at least five losses as compared to those with two or three losses (37 and 26%, respectively).[24]

In a study that looked at Caucasian couples with RM, it was shown that when either partner carried more than one thrombophilic mutation allele, the relative risk of miscarriage in a future untreated pregnancy is 1.9 (95% confidence interval 1.3-2.8); notably, the miscarriage rate was higher when it is the male that carried the

thrombophilic mutations.[25] However, the numbers of patients with thrombophilic mutations in this study were small, further validation is needed.

Men with oligospermia or azoospermia have a high risk of Y-chromosome microdeletion, with an estimated rate of 8-18% in this population of men.[26, 27] This knowledge prompted the investigation of Y-chromosome microdeletions in males from couples with unexplained RM, and indeed it was found that there was a higher rate of Y-chromosome microdeletions (~82%) in the RM group as compared to controls.[28]

Paternal aging comes with poorer sperm quality, increased rates of miscarriage, and an increase in birth defects. One study found that the rate of miscarriage was increased in males that were >40 years old.[29] In another study on 97 non-smoking men, a strong association was found between paternal age and the sperm DNA fragmentation index, which is associated with reduced fertility and miscarriage.[30]

Maternal factors

The assessment of maternal factors is performed as summarized in Table 1.2 with the goal of identifying in the female anatomical abnormalities, antiphospholipid syndrome, hormonal imbalances, thrombophilia, infection, and constitutive aneuploidies or structural chromosomal abnormalities.[31] In the majority of patients with RM, either subclinical abnormalities or no abnormalities at all can be identified even after comprehensive clinical and laboratory evaluations, and such patients are diagnosed with RM of unexplained clinical origin.

Table 1.2. Assessment of maternal factors

GENETIC STUDIES	EVALUATION OF PELVIC ANATOMY DAY 7-12
Parental karyotype	HSG :
Patient's :	Pelvic ultrasound :
Partner's :	PCO
POC's karyotype :	Mass
ROUTINE PRENATAL LABS	ENDOCRINLOGY
CBC : Hgb Plat Ferritin	TSH
Hgb electrophoresis	Thyroid antibodies
G6PD	2H OGTT : / /
Blood group Rh Abs	Ins. levels / /
Hep B	Day 2 – 5
Hep C	DHEAS
HIV	FSH
Parvo	LH
Rubella	Prolactin
Toxo	If PCO : Estradiol
VDRL	Free Testosterone
CMV	DAY 21 Progesterone
THROMBOPHILIA WORK-UP	
Immune : ANA	Thrombosis : APCR
RF	AT1II
SCAR	Prot C
LAC	Prot S
Coag	Factor VIII
Homocysteine	Cervical / vaginal cultures
Factor V Leiden	Gen'l GBS
Prothrombin mutation	GC Chlamydia
MTHFR	Ureaplasma Mycoplasma
Psychological support	

The full names of the tests are provided in the abbreviations section.

Anatomic abnormalities

Congenital and acquired uterine anomalies are found in 10% to 15% of women with RM compared with 7% of all reproductive-aged women.[32] Evaluation for uterine abnormalities is commonly performed for patients with RM and may include a hysterosalpingogram, saline infusion sonogram, ultrasound, hysteroscopy, or MRI. Uterine anatomical abnormalities are uterine septum, bicornuate uterus, intra-cavity lesions, and fibroids if at least 5 cm. Though there are no randomized controlled trials showing that surgical intervention decreases the subsequent miscarriage rate, the general consensus is that correction by hysteroscopy should be considered because it could potentially improve the outcome of subsequent pregnancies.[33] As an interesting side note, if an anatomical abnormality is present in a patient, it is important to evaluate her renal system because renal and uterine abnormalities are often associated.[34]

Antiphospholipid syndrome

Antiphospholipid syndrome is found in 5-20% of patients with RM.[35] Common tests for evaluation include lupus anticoagulant, anticardiolipin antibody, and anti B-2 glycoprotein I.[36] The reason behind testing these antibodies is that they can have a severe impact on the developing trophoblast: they can inhibit villous cytotrophoblasts differentiation and extravillous cytotrophoblasts invasion into the decidua, induce syncytiotrophoblast apoptosis, and initiate maternal inflammatory pathways in the syncytiotrophoblastic surface.[37-40]

Thyroid hormone

Untreated maternal hypothyroidism (high thyroid stimulating hormone associated with decreased levels of the T4 hormone) is associated with adverse pregnancy outcomes, including miscarriage, premature birth, low birth weight, and gestational hypertension.[41, 42] The most prevalent cause of hypothyroidism in pregnant women, is chronic autoimmune thyroiditis, which is known as Hashimoto's thyroiditis and affects ~0.5% of patients.[43] Subclinical hypothyroidism (high thyroid stimulating hormone but normal levels of T4) is also associated with adverse pregnancy outcomes, which include preterm deliveries, increased neonatal intensive care admissions, and neonatal distress after delivery.[44] Current guidelines recommend T4 replacement for patients with high TSH levels.[45]

Progesterone

Progesterone is a steroid hormone involved in the menstrual cycle, pregnancy, and embryogenesis. It triggers changes in the endometrium that prepare it for the embryo during the implantation window. Progesterone also helps to maintain a pregnancy, and to augment uterine receptivity by acting on growth factors and by regulating the production of cytokines Th1 and Th2. Specifically, it down-regulates Th1 and increases Th2, which is believed to be important for a healthy pregnancy.[46] Thus, it is thought that insufficient ovarian secretion of progesterone could cause RM. However, a big randomized trial of progesterone in women with RM (n=1568) found no difference between the progesterone group and the placebo group.[47]

Prolactin

Prolactin is commonly measured in women with RM because elevated prolactin levels are associated with ovulatory dysfunction. Excessive prolactin secretion decreases the pulsatile release of GnRH thus impairing the pituitary production of follicle stimulating hormone (FSH) and luteinizing hormone (LH), both of which are crucial for ovarian function. A 1998 randomized trial that looked at the effect of bromocriptine, a dopamine agonist that is a prolactin-reducing agent, on hyperprolactinemic patients with RM found an improved live birth rate in the treated group (85.7% versus 52.4%, $p<0.05$).[48] However, a more recent study provides contradictory data. This group looked at pregnancy outcomes in women exposed to dopamine agonists (the main dopamine agonist used was bromocriptine), using the EFEMERIS data (cohort of 57,408 pregnant women living in South West France). Their study group was made up of 183 women exposed to dopamine agonists and 366 age-matched controls. Interestingly, they found that exposure to dopamine agonists was associated with an increased risk of pregnancy loss [prevalence odds ratio (POR) = 3.7; 95 % CI 1.8–7.4] and preterm birth (POR = 3.6; 95 % CI 1.5–8.3).[49]

Polycystic ovarian syndrome

Polycystic ovary syndrome (PCOS) occurs in about 10% of reproductive-aged women.[50] The Rotterdam criteria for the diagnosis of PCOS involves having two out of three criteria, namely, PCO on ultrasound, irregular or absent menstrual cycles, and evidence of hyperandrogenism.[51] PCO is the most commonly identified

ultrasound abnormality in women with RM[52] and is well known to be associated, with or without hypersecretion of luteinizing hormone, with an increased rate of miscarriages.[53-55] The exact cause of PCOS is still unknown. Clinical presentation includes anovulation, menstrual disturbances, and hyperandrogenism. Obesity is commonly found in patients with PCOS and is associated with resistance to ovulation induction. As such, the primary treatment for obese women with PCOS is weight loss, and it has been shown to help menstrual disturbances, shorten the time to conception and reduce adverse obstetric risks.[56] Furthermore, some authors argue that insulin resistance explains the association between obesity, PCOS, and RM.[57] Metformin is an insulin-sensitizing drug; metformin treatment of PCOS patients decreases insulin resistance, thus improving ovulation cycles and, therefore, conception rates in infertile women.[58] Though some have found that metformin treatment can be beneficial in terms of reducing the miscarriage rate,[59] other studies, as shown by this review, [50] concluded that Metformin does not improve live birth rate or reduce miscarriage rate and should no longer be considered as an option for ovulation induction.

Thrombophilia

Thrombophilia is a condition where the blood has an increased tendency to venous thrombosis (a blood clot that forms in the veins), which is associated with an increased risk of sporadic and recurrent miscarriages.[60, 61] Tests to evaluate inherited thrombophilia disorders include DNA tests for Factor V Leiden and prothrombin gene promotor mutation, and biochemical test for deficiencies in

proteins C and S, all of which will be discussed in further detail in the section 1.5 on the genetics of RM in humans.

Infection

Some pathogens are known to increase the risk of miscarriage. These pathogens include malaria, brucellosis, cytomegalovirus, human immunodeficiency virus, influenza virus, syphilis, rubella, bacterial vaginosis and other vaginal infections with various microorganisms. The effects of Chlamydia trachomatis, Toxoplasma gondii, human papillomavirus, herpes simplex virus, parvovirus B19, Hepatitis B, and polyomavirus BK infections remain controversial, as some studies indicate increased miscarriage risk and others no. Finally, Q fever, adeno-associated virus, Bocavirus, Hepatitis C, and Mycoplasma genitalium infections do not appear to affect pregnancy outcome.[62]

Environment, life style, and risk factors

Results from a UK-population based case-control study on 603 women aged 18 to 55 years who had a first trimester miscarriage and 6116 controls found that the following factors were independently associated with an increased risk of miscarriage: high maternal age – odds of a miscarriage rise sharply after the age of 35, previous miscarriage, termination of pregnancy, infertility, assisted conception, low pre-pregnancy body mass index, regular or high alcohol consumption, feeling

stressed (including trend with number of stressful or traumatic events), high paternal age, and changing partner. Previous live birth, nausea, vitamin supplementation, and eating fresh fruits and vegetables daily were associated with reduced risk.[63]

Genetics of RM in humans

The evidence of a genetic susceptibility to RM is based on the following observations. The prevalence of RM is 2-7 times higher among first-degree blood relatives of patients with RM as compared to the control from the general population.[64] In addition, population-based studies indicated that the frequency of miscarriage is almost doubled in the siblings of patients with idiopathic RM.[65, 66] Approximately half of sporadic early miscarriages (<12 weeks of gestation) are caused by chromosomal abnormalities in the product of conception, and cytogenetic studies have shown that the majority of these abnormalities are numerical chromosomal abnormalities.[67] In RM, however, euploid miscarriages are more common, and this suggests an association of RM with factors that are not related to chromosomal abnormalities, such as uterine abnormalities, antiphospholipid syndrome, and thrombophilia.

The high percentage of women whose miscarriages are due to chromosomal abnormalities emphasizes the importance of cytogenetic evaluation, not only for diagnostic purposes, but also for the invaluable psychological benefit of identifying the cause of the miscarriage, for both sides, the patient's and the clinician's.

Karyotype abnormalities in miscarriages

The conventional technique for looking at chromosomal abnormalities is by karyotyping the POCs and the morphological examination of the chromosomes to identify numerical or structural abnormalities. Through a review that I performed on cytogenetic studies on large cohorts of sporadic miscarriages, I identified chromosomal abnormalities in 51.5% of the pooled miscarriages (total = 6491 miscarriages); of these, 57.8% had a trisomy, 16.2% were polyploid (triploid or tetraploid), 14.6% had monosomy X, 6.4% had a structural abnormality, and 5% had other abnormalities (including double, triple, and quadruple trisomies, as well as mosaicism).[68-80] The compiled data is shown in table 1.3.

I also compiled data about cytogenetic abnormalities in the POCs of patients with RM from several studies on large cohorts and summarized them in table 1.4. Of a total of 995 successfully analyzed miscarriages 43.8% were found aneuploid; of these, 63.1% had a trisomy, 17.2% were polyploidy, 7.1% had monosomy X, and 2.5% had a structural abnormality, and 10.1% had other abnormalities (including double, triple, and quadruple trisomies, as well as mosaicism)(Table 1.4).[74, 81-84]

These results demonstrate that euploid miscarriages are more common in patients with RM (56.2% of a total of 995 POC) than in patients with sporadic miscarriages (48.5% of a total of 6491 POC) (p-value=0.000007), suggesting the association of non-cytogenetic factors with RM.

Table 1.3. Summary of studies on genetic abnormalities in the POC of patients with sporadic miscarriages.

Reference	**Total number of samples** n	**Euploid** n (%)	**Aneuploid** n (%)	**Trisomy** n (%)*	**Polyploidy** n (%)*	**Monosomy X** n (%)*	**Structural abnormality** n (%)*	**Other** n (%)*
Hassold et al., 1980	1000	537 (54)	463 (46)	206 (44.5)	103 (22.2)	112 (24.2)	20 (4.3)	22 (4.8)
Eiben et al., 1990	746	370 (49.6)	376 (50.4)	229 (60.9)	62 (16.5)	40 (10.6)	18 (4.8)	27 (7.2)
Lomax et al., 2000	253	98 (39)	155 (69)	111 (72)	25 (16)	12 (8)	7 (5)	0
Fritz et al., 2001	57	16 (28)	41 (72)	28 (68)	7 (17.1)	4 (9.8)	1 (2.4)	1 (2.4)
Jobanputra et al., 2002	52	22 (42)	30 (58)	17 (57)	6 (20)	2 (7)	0	5 (17)
Nagaishi et al., 2004	347	151 (43.5)	196 (56.5)	120 (61.2)	32 (16.3)	24 (12.2)	13 (6.6)	7 (3.6)
Sullivan et al., 2004	133	77 (58)	56 (42)	53 (63)	12 (21)	5 (9)	3 (5)	0
Bruno et al., 2006	67	38 (57)	29 (43)	17 (59)	3 (10)	2 (7)	7 (24)	0
Diego-Alvarez et al. 2007	102	62 (61)	40 (39)	24 (60)	5 (13)	6 (15)	1 (3)	4 (10)
Menten et al., 2009	71	55 (76)	16 (23)	9 (53)	3 (18)	2 (12)	2 (12)	0
Robberecht et al., 2009	77	55 (71)	22 (29)	10 (45)	5 (23)	6 (27)	1 (5)	0
Zhang et al., 2009	92	37 (40)	55 (60)	36 (66)	8 (15)	5 (9)	2 (4)	4 (7)
Shearer et al., 2011	3361	1627 (48)	1734 (52)	1074 (62)	278 (16)	260 (15)	135 (8)	89 (5)
Choi et al., 2014	164	81 (49.4)	83 (50.6)	52 (62.7)	6 (7.2)	12 (14.5)	6 (7.2)	7 (8.4)
Total	6491	3149 (48.5)	3342 (51.5)	1933 (57.8)	543 (16.2)	487 (14.6)	213 (6.4)	166 (5)

Other includes: double, triple, quadruple trisomies, mosaicism, autosomal monosomies, and one trisomy plus a balanced translocation.

* indicates percentage of aneuploidies.

Table 1.4. Summary of studies on genetic abnormalities in POCs from patients with RM.

Reference	RM definition	Total number of samples	Euploid	Aneuploid	Trisomy	Polyploidy	Monosomy X	Structural abnormality	Other
		n	n (%)	n (%)	n (%)*	n (%)*	n (%)*	n (%)*	n (%)*
Stern et al., 1996	≥ 2	94	40 (42.6)	54 (57.4)	45 (83.3)	9 (16.7)	0	0	0
Ogasawara et al., 2000	≥ 2	234	114 (48.7)	120 (51.3)	63 (52.5)	18 (15)	5 (4.2)	0	34 (28.3)
Carp et al., 2001	≥ 3	125	89 (71.2)	36 (28.8)	24 (66.7)	5 (13.9)	5 (13.9)	2 (5.5)	0
Stephenson et al., 2002	≥ 3	420	225 (53.6)	195 (46.4)	122 (62.6)	37 (19)	18 (9.2)	8 (4.1)	10 (5.1)
Sullivan et al., 2004	≥ 2	122	91 (74.6)	31 (25.4)	21 (67.7)	6 (19.4)	3 (9.7)	1 (3.2)	0
Total		995	559 (56.2)	436 (43.8)	275 (63.1)	75 (17.2)	31 (7.1)	11 (2.5)	44 (10.1)

Other includes: double, triple, quadruple trisomies, mosaicism, and autosomal monosomy.
* indicates percentage of aneuploidies.

Copy number variations in miscarriages

Copy number variants (CNVs) are submicroscopic chromosomal changes (longer than 1kb)[85] that cannot be detected by conventional morphological karyotype analysis of chromosomal bands, but are detectable by array-comparative genome hybridization (array-CGH). CNVs could contribute to pregnancy loss if they are very close to or if they overlap a gene/genes that play a role in pregnancy and affect their expression. According to a recent study that looked at CNVs in 101 euploid miscarriages, the pathogenicity of a CNV was found to depend on its size (>1 Mb), whether it is *de novo* or not, and whether it affects the expression of the gene(s) that are close to it and whether the affected gene(s) are important for normal pregnancy.[86] In this study, the authors compared rare CNVs to common CNVs and found that rare CNVs had a significantly higher mean gene density. These rare CNV genes were twice as likely to have an abnormal phenotype in mouse knockout models, as compared to common CNV genes.[86]

In conclusion, CNVs can be detected in 5% of miscarriages that have a normal karyotype.[87] However, it is still unknown if these CNVs are clinically significant or not since only few studies have been done.

Genes associated with miscarriages

Variants in approximately 100 genes have been shown to be associated with RM in some populations and studies and are recapitulated in table 1.5.[88] However, many of these associations were not replicated when tested in other populations or in meta-

analyses.[89, 90] We believe that the reasons of the lack of replications of these studies are mainly the wide clinical heterogeneity of the entity of RM, the lack of systematic comprehensive evaluations of studied patients, the lack of standardized criteria of evaluations that are used in all studies, and the small sizes of the study cohorts relative to the heterogeneity of this entity. Consequently, most of these associations were not replicated and are not used in molecular DNA testing of patients with RM with the exception of variants in two genes, Factor V and prothrombin, which play roles in thrombophilia.

The variant in Factor V is called Leiden allele and is the result of a substitution from an A to a G at nucleotide position 1601 (p.Arg534Gln) (NM_000130.4). The prevalence of this variant in the general population varies between 2-5% according to ethnic groups.[91] According to a meta-analysis, Factor V Leiden is associated with a 2.9 fold (95% CI 2.0-4.3) increased risk of severe preeclampsia, and a 4.8 fold (95% CI 2.4-9.4) increased risk of fetal growth retardation.[92] According to another meta-analysis, this allele significantly increase the risk for early first-trimester recurrent loss (odds ratio 2.1) and late recurrent and non-recurrent fetal loss (odds ratios 7.8 and 3.2, respectively).[60]

The variant in the prothrombin gene is a substitution of a G to an A at position 20210 of the genomic DNA (NG_008953.1), which is in the promoter region of the gene. At the functional level, this variant leads to an increased prothrombin level,[93] which is associated with a higher risk of thromboembolism or the obstruction of a vessel by a blood clot.

Table 1.5. Genes associated with recurrent miscarriages.

Role of gene	Gene	Full gene name	Reference
Chromosome segregation	SYCP3	Synaptonemal complex protein 3	Bolor et al. (2009), Hanna et al. (2011), Mizutani et al. (2011)
Detoxification system	AHR	Aryl hydrocarbon receptor	Saijo et al. (2004b)
	ARNT	Aryl hydrocarbon receptor nuclear translocator	Sullivan et al. (2006)
	CYP1A1	Cytochrome P450 family 1 subfamily A member 1	Parveen et al. (2010), Saijo et al. (2004b)
	CYP1A2	Cytochrome P450 family 1 subfamily A member 2	Saijo et al. (2004b), Sata et al. (2005)
	CYP1B1	Cytochrome P450 family 1 subfamily B member 1	Saijo et al. (2004b)
	CYP2D6	Cytochrome P450 family 2 subfamily D member 6	Parveen et al. (2010), Suryanarayana et al. (2004)
	GSTM1	Glutathione S-transferase mu 1	Parveen et al. (2010), Sata et al. (2003a)
	GSTP1	Glutathione S-transferase pi 1	Parveen et al. (2010), Zusterzeel et al. (2000)
	GSTT1	Glutathione S-transferase theta 1	Parveen et al. (2010), Sata et al. (2003a)
	NAT2	N-acetyltransferase 2	Hirvonen et al. (1996)
Hormonal regulation	AR	Androgen receptor	Karvela et al. (2008b), Su et al. (2011a)
	hCG beta	Beta-human chorionic gonadotropin	Rulletal.(2008)
	CYP17A1	Cytochrome P450 family 17 subfamily A member 1	Litridis et al. (2011), Sata et al. (2003b)
	CYP19A1	Cytochrome P450 family 19 subfamily A member 1	Cupisti et al. (2009), Suryanaryana et al. (2007)
	PROGINS, ESR1/2	PR variant, Estrogen receptor 1/2	Su et al. (2011a), Traina et al. (2011)
Immune response	CCR5	C-C motif chemokine receptor 5	Parveen et al. (2009a, 2011b)
	CTLA4	Cytotoxic T-lymphocyte associated protein 4	Tsai et al. (1998), Wang et al. (2005)
	CX3CR1	C-X3-C motif chemokine receptor 1	Parveen et al. (2011b)
	HLA-A, B	Major histocompatibility complex, class I, A, B	Beydoun and Saftlas (2005), Christiansen et al. (1989), Kolte et al. (2010)
	HLA-C	Major histocompatibility complex, class I, C	Beydoun and Saftlas (2005), Faridi and Agrawal (2011), Hiby et al. (2010), Moghraby et al. (2010)
	HLA-E	Major histocompatibility complex, class I, E	Kanai et al. (2001), Mosaad et al. (2011), Steffensen et al. (1998)
	HLA-G	Major histocompatibility complex, class I, G	Aruna et al. (2010), Cecati et al. (2011), Hviid et al. (2004), Kolte et al. (2010), Ober et al. (2003)
	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	Takakuwa et al. (1999)
	HLA-DQA1, HLA-DQB1	Major histocompatibility complex, class II, DQ alpha 1, beta 1	Aruna et al. (2011), Kruse et al. (2004), Steck et al. (1995)
	HLA-DR	Major histocompatibility complex, class II, DR	Beydoun and Saftlas (2005), Christiansen et al. (1999), Kolte et al. (2010), Kruse et al. (2004)
	INDO	Indoleamine 2,3-dioxygenase 1	Amani et al. (2011)
	KIR	Killer cell immunoglobulin like receptor	Faridi et al. (2009), Hiby et al. (2008), Witt et al. (2004)
	MBL	Mannose binding lectin	Baxter et al. (2001)

Role of gene	Gene	Full gene name	Reference
Inflammation	IFNG	Interferon gamma	Bombell and McGuire (2008), Daher et al. (2003)
	IL1B	Interleukin 1 beta	Bombell and McGuire (2008)
	IL1RN	Interleukin 1 receptor antagonist	Choi and Kwak-Kim (2008)
	IL1R1	Interleukin 1 receptor type 1	Traina et al. (2011)
	IL4	Interleukin 4	Kamali-Sarvestani et al. (2005), Saijo et al. (2004a)
	IL6	Interleukin 6	Bombell and McGuire (2008), Daher et al. (2003)
	IL10	Interleukin 10	Bombell and McGuire (2008), Daher et al. (2003)
	IL12B	Interleukin 12B	Ostojic et al. (2007)
	IL18	Interleukin 18	Al-Khateeb et al. (2011), Naeimi et al.
	IL21	Interleukin 21	Messaoudi et al. (2011)
	TNFα	Tumor necrosis factor alpha	Bombell and McGuire (2008), Daher et al. (2003)
	TNFβ	Tumor necrosis factor beta	Kamali-Sarvestani et al. (2005), Prigoshin et al .(2004)
	TNFR1	TNF receptor superfamily member	Yu et al. (2007)
Mitochondrial function	Mutational burden		Kaare et al. (2009b), Seyedhassani et al. (2010), Vanniarajan et al. (2011)
Placental	ACP1	Acid phosphatase 1, soluble	Gloria-Bottini et al. (1996)
	ADA	Adenosine deaminase	Nicotra et al. (1998)
	ADRA2B	Adrenoceptor alpha 2B	Galazios et al. (2011)
	ANGPT2	Angiopoietin 2	Pietrowski et al. (2003)
	CD14	CD14 molecule	Karhukorpi et al. (2003)
	EG-VEGF	Endocrine gland-derived vascular endothelial growth factor	Su et al. (2010)
	H19	H19, imprinted maternally expressed transcript	Ostojic et al. (2008)
	IGF-2	Insulin like growth factor 2	Ostojic et al. (2008)
	KDR	Kinase insert domain receptor	Su et al. (2011b)
	MCP	CD46 molecule (old name: membrane cofactor protein)	Heuser et al. (2011)
	MMP9	Matrix metallopeptidase 9	Singh et al. (2012)
	NOS3	Nitric oxide synthase 3	Karvela et al. (2008a), Parveen et al.
	P53	Tumor protein p53	Coulam et al. (2006b), Kaare et al. (2009a), Pietrowski et al. (2005), Tang
	PAPPA	Pappalysin 1	Suzuki et al. (2006)
	PGM1	Phosphoglucomutase 1	Bottini et al. (1983), Nicotra et al. (1982)
Thrombosis and cardiovascular	ACE	Angiotensin I converting enzyme	Goodman et al. (2009b), Zhang et al. (2011)
	ACHE	Acetylcholinesterase (Cartwright blood group)	Parveen et al. (2009b)
	AGT	Angiotensinogen	Goodman et al. (2009b), Hefler et al.
	ANXA5	Annexin A5	Bogdanova et al. (2007), Miyamura et al. (2011)
	APOB	Apolipoprotein B	Hohlagschwandtner et al. (2003), Yenicesu et al. (2009)
	APOE	Apolipoprotein E	Bianca et al. (2010), Goodman et al.
	AT1R	Angiotensin II Receptor Type 1 (also known as AGTR1)	Buchholz et al. (2004), Fatini et al. (2000)
	EPCR	Protein C receptor (also known as PROCR)	Dendana et al. (2012), Kaare et al. (2007)

Role of gene	Gene	Full gene name	Reference
Thrombosis and cardiovascular	F2	Coagulation factor II, thrombin	Kovalevskyet al. (2004), Silver et al. (2010), Toth et al. (2008)
	F5	Coagulation factor V	Dudding and Attia (2004), Kovalevsky et al. (2004), Rey et al. (2003), Rodger et al. (2010), Toth et al. (2008)
	FGB	Fibrinogen beta chain	Goodman et al. (2006), Yenicesu et al
	F12	Coagulation factor XII	Sotiriadis et al. (2007), Walch et al.
	F13A	Coagulation factor XIII	Coulam et al. (2006a), Sotiriadis et al. (2007), Yenicesu et al. (2009)
	GPIa	Glycoprotein Ia	Gerhardt et al. (2005)
	GPIIIa	Glycoprotein IIIa	Ivanov et al. (2010), Pihusch et al. (2001), Yenicesu et al. (2009)
	HMOX1	Heme oxygenase 1	Denschlag et al. (2004)
	JAK2	Janus kinase 2	Dahabreh et al .(2009)
	MTHFD1	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1	Crisan et al. (2011)
	MTHFR	Methylenetetrahydrofolate reductase	Nelen et al. (2000), Ren and Wang (2006), Toth et al. (2008)
	PAI-1	Serpin family E member 1 (new name: SERPINE1)	Buchholz et al. (2003), Goodman et al. (2009b), Sotiriadis et al. (2007)
	PZ	Protein Z, vitamin K dependent plasma glycoprotein	Dossenbach-Glaninger et al. (2008), Topalidou et al. (2009)
	SELP	Selectin P	Dendana et al. (2011)
	TAFI	Carboxypeptidase B2 (also known as CPB2)	Masini et al. (2009)
	TGFB1	Transforming growth factor beta 1	Prigoshin et al. (2004), von Linsingen et al. (2005)
	TM	Thrombomodulin (also known as	Kaare et al. (2007)
	TSER	Thymidylate synthase enhancer region (new gene symbol: TYMS)	Kim et al. (2006a)
	VEGF	Vascular endothelial growth factor	Papazoglou et al. (2005), Traina et al.
	ZPI	Serpin family A member 10 (also known as SERPINA10)	Alsheikh et al. (2012)

Table adapted from Laan et al., 2012

Blood coagulation

Figure 1 provides an overview of the blood coagulation process to help understand the roles of Factor V and prothrombin genes. Blood coagulation is regulated by two major proteins, protein C and protein S. Protein C gets activated by binding to thrombin and becomes Activated Protein C (APC). Protein S serves as a cofactor for APC and binds to it; together they degrade Factor V and consequently decrease thrombin formation. The Leiden variant makes Factor V resistant to degradation by APC, and thus results in increased thrombin formation.

Prothrombin is the precursor of thrombin, which plays a key role in coagulation in two ways. First, thrombin converts fibrinogen to fibrin, the main component of a blood clot. Second, prothrombin activates Factor V, which leads to thrombin formation. A deficiency in proteins C and S lead to an increase in the generation of thrombin. Lastly, antithrombin III is an inhibitor of thrombin, both directly and indirectly. In the assessment of patients with RM, two variants in Factor V and prothrombin gene promoter are tested at the molecular level and Proteins S and C are tested at the biochemical level.[94]

Genes that cause RM

So far, only one gene that codes for the synaptonemal complex protein 3 (SYCP3), a component of the synaptonemal complex that forms between homologous chromosomes in prophase of MI and play a role in female and male meiosis, was shown to cause RM based on the presence of heterozygous protein truncating

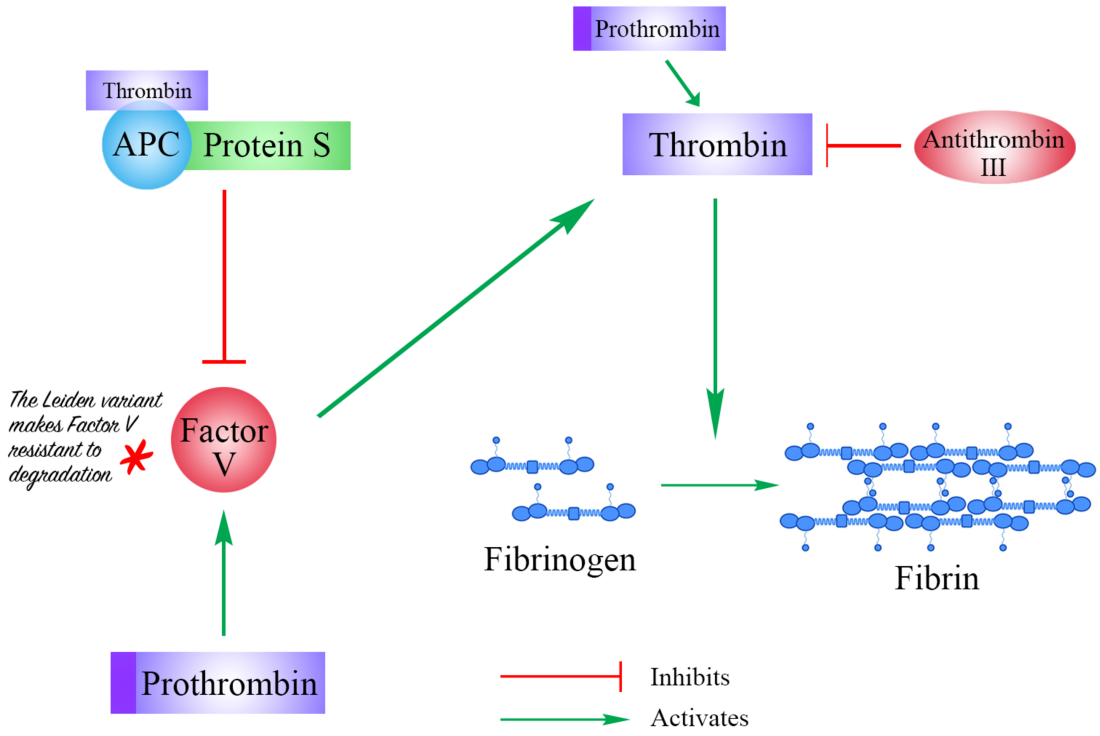

Figure 1. Overview of the blood coagulation process. Protein C gets activated by binding to thrombin and becomes Activated Protein C (APC), with Protein S as a cofactor. Together, they degrade Factor V and consequently decrease thrombin formation. The Leiden variant makes Factor V resistant to degradation by APC, and thus results in increased thrombin formation.

mutations in SYCP3 in two patients with RM.[95] However, subsequent studies did not replicate these findings in other cohorts[95-99] and populations indicating that SYCP3 mutations may underlie a minority of cases of RM.

The main factors that have hampered the identification of causative or susceptibility genes for RM lies in the difficulty in dividing the patients into homogeneous categories and the high genetic heterogeneity of this clinical entity.

Rational and objectives of my project

RM is a heterogeneous entity, which makes it impossible to tease out its aetiology without subdividing it into smaller homogeneous categories. Thus, the main objective of my project was to identify a subset of patients with a rare, recurrent, and unique mechanism that is responsible for their RM and to identify the gene that causes their recurrent rare mechanism.

Chapter 2: Identification of a patient with recurrent triploidy of maternal origin

Introduction

A miscarriage is the spontaneous arrest of embryonic development before 20 weeks of gestation[88] and it affect 15% of clinically recognized pregnancies (for review, see Stephenson 2007).[31, 100] Recurrent miscarriages (RM) are defined by the occurrence of at least two pregnancy losses and affect 1% to 5% of couples trying to conceive. RM are clinically and genetically highly heterogeneous.[3]

At the clinical level, various abnormalities such as infections, parental karyotype anomalies, endocrine, pelvic anatomical, thrombophilic, and autoimmune abnormalities may be found in women with RM. However, these abnormalities account for only 50% of the cases and in the remaining cases, no abnormalities at all can be identified in the patients even after comprehensive clinical and laboratory evaluations.[88, 101-103] Such patients are diagnosed with RM of unexplained clinical origin.

At the genetic level, there are few known genes that play causal roles for RM in humans, and they only explain a very small proportion of the cases. Variants in approximately 100 genes have been shown to be associated with RMs in some populations and studies, but many of these associations were not replicated when tested in other populations or in meta-analyses.[89, 90] The main factors that have hampered the identification of causative or susceptibility genes for RM lies in the difficulty in dividing the patients into homogeneous categories and the high genetic heterogeneity of this clinical entity.

In an attempt to further categorize the entity of RM and divide the patients into additional categories based on the mechanisms leading to their RM, we first used flow cytometry to assess the ploidy of 96 POCs from 54 patients. We identified six triploid POCs (6%), of which three are from unrelated patients and three are from the same patient. Next, we determined the parental origin of the six triploidies and found that they are all of maternal origin, one resulting from MI failure and five from MII failure. We reviewed the medical files of the four patients with triploidies and found that three of them had polycystic ovarian syndrome (PCOS), pointing to a possible association between PCOS and triploidy of maternal origin. In one of these patients, we found three triploid miscarriages that are all due to an error in MII. Identifying a patient with three recurrent triploidies of maternal origin that are all due to one recurring mechanism is a unique opportunity to search for a genetic predisposition for this condition.

Methods

Patients

This study was approved by the McGill Institutional Review Board. All patients provided written consent to participate in the study. A total of 123 patients were recruited for this study from the Poor Pregnancy Outcome Clinic at the Royal Victoria Hospital of the McGill University Health Centre between May 2006 and August 2014 (established and ran by Dr. William Buckett and his team). All patients had at least three miscarriages (≥3 miscarriages) and no hydatidiform moles. The patients answered a comprehensive questionnaire about their medical and family

histories and were comprehensively evaluated by their physician according to the evaluation summary provided in Table 1.2. The medical histories of all patients were systematically reviewed with their referring physicians and the patients were classified into one of the following aetiologies: 1) constitutive chromosomal abnormalities in parental blood, 2) pelvic anatomical abnormalities, 3) antiphospholipid syndrome, and 4) thrombophilic abnormalities.[104, 105] In 74% of our cases, either subclinical abnormalities or no abnormalities could be identified even after comprehensive clinical and laboratory evaluations, and such patients were classified into a fifth category, 5) clinically unexplained.

Karyotyping was performed using PHA stimulation of blood cells and classical cytogenetic analysis. Uterine anatomical abnormalities were diagnosed for cases with uterine septum or fibroids of at least 5 cm in any dimension.[106, 107] Diagnosis of antiphospholipid syndrome was based on the presence of lupus anticoagulant antibodies and/or anticardiolipin, and β2-Glycoprotein IgG and/or IgM antibodies, on two positive tests that are at least 12 weeks apart.[108] Diagnosis of thrombophilia was based on the presence of the Factor V Leiden or prothrombin DNA variants, and/or abnormal values in any of the following tests: activated protein C resistance, antithrombin III assay, levels of protein C, protein S, and Factor VIII.

The most relevant medical finding at the basis of the diagnosis and classification of each patient is provided in Table 2.1.

Paraffin-embedded POCs were retrieved from various pathology departments.

Table 2.1. Clinical information for our study cohort of patients with RSAs, showing reproductive history, RSA category (primary or secondary), final diagnosis, and additional information.

Patient ID	Reproductive History	Primary or Secondary RM	Final Diagnosis in Category	Additional Information
Parental cytogenetic abnormality				
828	5 SA, 2 LB	Primary	Patient karyotype: 46,X,fragile(X)(q27~q28)	
949	3 SA, SA (45,X), IVF-PGS-SA, SA	Primary	Patient karyotype: 45,XX,der(13;14)(q10;q10)	
932	LB, 4 SA	Secondary	Patient karyotype: 45,X[4]/47,XXX[3]/46,XX[59]	Borderline ovarian functions
1014	LB, EP, 2 SA, SA, SA(46,XX), LB	Secondary	Patient karyotype: 45,X[3]/46,XX[27]	Prothrombin (het)
Unexplained				
554	5 SA, 6 failed IVF, donated eggs- LB-twins	Primary	Unexplained	PCOS
1138	4 SA, BO, 4 SA, LB	Primary	Unexplained	PCOS
1234	SA, CP, BO, 3 IVF-SA, IVF-PGS-LB	Primary	Unexplained	PCOS
1091	SA, LB, 3 SA, LB	Primary	Unexplained	PCOS
1239	5 SA	Primary	Unexplained	PCOS
1299	LB, eTOP, 2 SA, 2 EP, 4 failed IVF-ET, LB	Secondary	Unexplained	PCOS
693	BO, 2 CP, CP, SA, CP, EP, 2 CP	Primary	Unexplained	PCO
1154	Infertility, IUI-LB, 3 SA, F-IVF, IUFD (21w), LB (36w, IUGR)	Secondary	Unexplained	PCOS, MTHFR het
874	3 SA, LB, 5 SA, 2 LB	Primary	Unexplained	PCOS, MTHFR het
1058	SA, LB, 4 SA	Primary	Unexplained	PCOS, MTHFR het
1009	4 SA	Primary	Unexplained	PCO, Chronic endometritis, MTHFR het
821	4 SA, 2 EP	Primary	Unexplained	Chronic endometritis, MTHFR het
809	3 SA, TOP(trisomy 18), LB	Primary	Unexplained	Chronic endometritis, MTHFR het
1303	7 SA	Primary	Unexplained	Chronic endometritis, MTHFR het
1017	6 SA	Primary	Unexplained	Dysmenorrhea, MTHFR het
1175	LB, SB (39w), 3 SA	Secondary	Unexplained	Cerebral stroke, placental thrombosis, 2x raised TSH
1309	3 SA, LB, SA, LB	Primary	Unexplained	PCOS, hypothyroidism (high TSH & anti-TPO)
565	SA, LB, 6 SA, IVF-PGD-LB-twins	Primary	Unexplained	Hashimoto disease
991	4 SA	Primary	Unexplained	Hashimoto disease, one fibroid (2.56x2.06x2.77)
1005	5 SA	Primary	Unexplained	Hashimoto disease
1300	LB, EP, LB, 4 SA	Secondary	Unexplained	Hypothyroidism on synthroid, fibroid (3.6x3.6)
1192	5 SA, PR	Primary	Unexplained	mildly hypothyroidism, on synthroid, MTHFR het
952	LB, 3 SA, 2 LB	Secondary	Unexplained	Hypothyroidism on synthroid

936	4 SA, TOP (47,XX+21), LB	Primary	Unexplained	Antinuclear antibody positive; MTHFR het
1326	eTOP, 4 SA, LB, SA, LB	Primary	Unexplained	ANA positive
1013	LB, 2SA, TOP (trisomy 21), SA, CP	Secondary	Unexplained	Raised rheumatoid factor, MTHFR het
947	4 SA, IUI-SA	Primary	Unexplained	MTHFR het
990	eTOP, EP, LB (ab), 2 SA, 2 LB	Primary	Unexplained	MTHFR het
806	SA, BO, 2 LB, SA, LB, 2 SA, LB, SA	Primary	Unexplained	MTHFR het
885	5 SA, 1 failed donated egg	Primary	Unexplained	MTHFR het
1366	9 SA	Primary	Unexplained	None
831	LB, 7 SA	Secondary	Unexplained	None
1339	LB, 4 SA	Secondary	Unexplained	None
2006	LB, 3 SA, LB	Secondary	Unexplained	None
946	LB, 4 SA, 2 failed IVF	Secondary	Unexplained	None
815	SA, SA, SA, SA, LB	Primary	Unexplained	None
1346	SA, 2 LB, 3 SA, LB	Primary	Unexplained	None
Antiphospholipid				
819	2 BO, LB, 2 SA, LB	Primary	Antiphospholipid syndrome	Lupus anticoagulant 2 times, 12 weeks apart
887	10 SA	Primary	Antiphospholipid syndrome	Anticardiolipin IgM 2* high, 12 weeks apart
1030	SA, 1 LB, 3 SA	Primary	Antiphospholipid syndrome	Anticardiolipin IgM 1 time high, PCOS
1083	2 eTOP, LB, ET, 2 SA	Secondary	Systemic lupus	Antithyroid antibodies, MTHFR het
Uterine anatomical abnormalities				
856	eTOP, 3 SA, LB	Primary	Uterine septum	MTHFR het
1155	3 SA, LB (33w), LB	Primary	Uterine fibroid (3 removed, largest 7.5x6.5x5.5 cm)	PCOS
1313	5 SA	Primary	Uterine fibroids (4 removed, largest 7.0x4.5x4.0 cm)	Hypothyroidism (high TSH & anti-TPO), on synthroid
Thrombophilias				
1016	4 SA, LB	Primary	Prothrombin mutation (het)	Raised rheumatoid factor, Antinuclear antibody positive
807	4 SA, LB	Primary	Factor V Leiden (het)	Leiden V het, MTHFR het
Incomplete evaluation				
1043	LB, 5 SA, EP	Secondary	Incomplete evaluation	
818	BO, LB (ab), EP, 2 BO, BO, 2*IVF-3 ET each time, BO, PR (US ab)	Primary	Incomplete evaluation	
1136	3 SA	Primary	Incomplete evaluation	
930	2 SA, LB, 2 SA, LB	Primary	Unexplained, evaluation not available	MTHFR het

SA stands for spontaneous abortion; LB, live birth; IVF, in vitro fertilization; PGS, prenatal genetic screening; EP, ectopic pregnancy; BO, blighted ovum; CP, chemical pregnancy; eTOP, elective termination of pregnancy; IUI, intra-uterine insemination; IUFD, intra-uterine fetal death; IUGR, intra-uterine growth restriction; PR, pregnant; PCOS, polycystic ovary syndrome; het, heterozygous; TSH, thyroid stimulating hormone; TPO, thyroid peroxidase; ANA, antinuclear antibody.

Oocyte collection

Patients undergoing in vitro fertilization or preimplantation genetic diagnosis were asked for consent to donate their spare immature oocytes for research. These patients are different from our cohort of patients with RM (table 2.1). Oocytes were retrieved by transvaginal ultrasound-guided retrieval 35-38 h following a single 10 000 IU hCG

injection, which is administered when two or more follicles have a diameter that is over 18 mm. The oocytes are then cultured in embryo culture medium (Life Global Group). Spare oocytes were donated for research on day 3 after collection.

Flow cytometry on formalin-fixed paraffin-embedded (FFPE) tissues

Flow cytometry was performed on formalin-fixed paraffin-embedded (FFPE) tissues. Cellular preparation for flow cytometry was performed according to a modified version of Hedley's protocol.[109] Two sections of 60 µm were cut from each FFPE block and placed in 15 ml Falcon tubes. The sections were deparaffinised twice for 10 minutes in 5 ml of xylene and rehydrated in a sequence of 5 ml of 100%, 100%, 95%, 70%, and 50% ethanol for 10 min, each, at room temperature, and washed twice in 10 ml Milli-Q water for 10 min. Five ml of cold (stored at 4°C) citrate solution, 10 mmol/L, pH 6.0) was added to each tube and the tubes were incubated at 80°C for 2 h. They were then allowed to cool to room temperature for 15 min and rinsed with PBS-1X. The proteins were digested in 1 ml of 5 mg/ml pepsin (Sigma) in 0.9% NaCl (adjusted to pH 1.5 with HCl) for 30 min with

intermittent vortexing every 10 min. The cellular suspension was then rinsed with PBS-1X and suspended in propidium iodide (PI) solution (0.1 mg/µl, Sigma-Aldrich, St Louis, MO) and 50 µl RNase (1 mg/ml) and incubated at 37°C for 30 min. Finally, they were filtered through a 48 µm mesh nylon filter (Les Industries Filmar, www.filmar.qc.ca) and analyzed using a BD FACS Canto II at the Immunophenotyping Core Facility of the McGill University Health Centre. Data files were analyzed using FCSalyzer (Wien, Austria), which is a program that makes it possible to filter out the debris, in a process called gating, that could potentially drown out/mask the important ploidy peaks.

Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization (FISH) was performed on 4 µm sections as a service by the laboratory of Professor U. Surti. Slides were hybridized with probes from the centromeres of three chromosomes, X, Y and 18, as previously described (Surti, et al., 2006). For each POC, more than 100 cells from different microscopic fields were scored with each probe.

Microsatellite genotyping

Depending on the amount of chorionic villi (CV) in the paraffin blocks, 5-12 serial 10 µm sections were prepared from the blocks that contained the largest amount of CV that are separated from maternal tissues. These sections were mounted on slides and stained with hematoxylin and eosin (H&E). Under a stereomicroscope, maternal

tissues that are in close proximity to the CV were removed and discarded while the CV were then removed from the slides using Kimwipes and forceps and used for DNA extraction using phenol-chloroform. An aliquot of the extracted DNA was loaded on an agarose gel to evaluate the quality and quantity of DNA to be used for microsatellite genotyping with the PowerPlex 16 HS System (Promega, Corporation, Fitchburg, Wisconsin, USA). The reaction consisted of short tandem repeat (STR) multiplex PCR assays that amplify DNA at 15 different STR loci and a fragment from the Amelogenin gene that distinguishes the X and Y chromosomes. In addition, 11 pericentromeric markers mapped at less than 5 Mb from the centromeres of several chromosomes were selected from the Marshfield genetic map (http://www.bli.uzh.ch/BLI/Projects/genetics/maps/marsh.html) and from previous studies [110] and used for the triploid digynic (i.e. two maternal sets of chromosomes and one paternal set) conceptions to determine whether the triploidies originated from failure of MI or MII, as previously described by Zaragoza et al.[110] Primer sequences for the pericentromeric markers are provided in Table 2.2. DNA from the POCs and their available parents was amplified and the PCR products were resolved by capillary electrophoresis using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Toronto Centre for Applied Genomics (http://www.tcag.ca). The data were analyzed with PeakScanner version 1.0 (Applied Biosystems, Foster City, CA, USA) and the POC alleles were compared to the parental alleles to determine their origin.

Table 2.2. Primer sequences for the pericentromeric markers used in simplex genotyping

Marker	Primer Sequence (5' to 3')	Annealing temperature
D1S534_F	AGCACATAGCAGGCACTAGC	58°C
D1S534_R	CGATTGTGCCACTACACAGT	58°C
D3S2462_F	TTAATCTGCCAACTTGTCTGG	55°C
D3S2462_R	TTTTCACCTGTGCTGTTGCT	55°C
D4S3355_F	CCATCAACCACCATGAGTAA	55°C
D4S3355_R	CCCTGCAAAAATGACTCTGT	55°C
D6S402_F	AACAACCATGCAGTGCT	58°C
D6S402_R	TCACAGGCAAACAACAA	58°C
D7S1485_F	ACTCCAGCCTGGGTGACAC	58°C
D7S1485_R	ATGATTCTCACCAGTGGCC	58°C
D8S1115_F	GGCCTAGGAAGGCTACTGTC	58°C
D8S1115_R	CACCATAATGTTTTCCACAGC	58°C
D11S1983_F	ATTCTGTGTCTAAAAACAGAAAAGA	55°C
D11S1983_R	TTACCAGGAAAGAGGGGAAT	55°C
D12S2080_F	TCTTGATAGCCTGCCCTATG	55°C
D12S2080_R	GGGCAAGGTATCAATCAGTG	55°C
D14S122_F	CCAGCCTGGGTGAGACTC	58°C
D14S122_R	CGTTCATGTACCACTGCATG	58°C
D18S869_F	TGTTTATTTGTTTGACTCAATGG	55°C
D18S869_R	GAGTGAATGCTGTACAAACAGC	55°C
D20S484_F	TATCAGGCCTCACCCTGG	55°C
D20S484_R	AAAAGAATAAGAAGCTCTAAAAGTG	55°C

F is for the forward primer and R is for the reverse primer. All of these markers are pericentromeric and lie within 5 Mb from the centromere.

Whole-exome sequencing

Whole-exome sequencing was performed at McGill University and Génome Québec Innovation Centre (Montreal, Canada) as previously described.[111, 112] Briefly, the exome was captured using the SeqCap EZ Exome v3.0 and Nextera Rapid Capture on 3 μg of genomic DNA from all the patients with triploid conceptions, as well as their available family members when available. Sequencing was carried out with pair-end 100 base reads on the Illumina Hiseq 2000 sequencer. Trimming was performed using Trimmomatic v 0.32. The reads were aligned to the Genome Reference Consortium Human genome build 37 with BWA-MEM 0.7.10.[113] Post-processing of the BAM files was done with GATKv3.2-2 Indel Realigner, Mark Duplicates, and Base recalibration Variants were called using the GATK HaplotypeCaller (v3.2-2).[114] The functional annotations were done using SnpEff v3.6 to GRCh37.75 and the annotations with Gemini version 0.16.3.

Confirmational Sanger sequencing

Selected variants in important candidate genes were confirmed by Sanger sequencing at Génome Québec Innovation Centre. Primers were designed, using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), to surround the variant in the gene of interest in order to confirm the identified variant by another method.

Gene expression analysis in oocytes by RT-PCR

Oocytes were collected from the McGill Reproductive Centre following patient consent. Under a stereomicroscope, the oocytes were transferred to droplets of acidic Tyrode's solution to carefully denude the oocyte by removing the zona pellucida and the surrounding cumulus cells, in order to eliminate the presence of DNA from somatic cells. The oocytes were then washed in PBS-1x containing 3% of bovine serum albumin, put in 5 µl of lysis buffer (0.5% NP-40, 10mM Tris, 10mM NaCl, 3 mM $MgCl_2$), and kept at -20 °C until use in RT-PCR. Finally, the cDNA was used to check for the expression of the genes of interest in denuded oocytes by PCR amplification.

Immunofluorescence on HeLa cells

Given that human oocytes are not easy to obtain and are therefore precious, we first tested our protocol and antibodies on HeLa cells. Four-well chamber slides (Ultident) were seeded with 10,000 HeLa cells per well and left overnight in culture at 37°C. In order to enrich for dividing cells, the cells were synchronized by a double-thymidine block.[115] Cells were treated with thymidine at a final concentration of 2 mM and kept in the incubator for 18 h, then washed with PBS-1x and incubated in Dulbeco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% of fetal bovine serum (FBS) (Invitrogen) for 9 h. They were treated again with thymidine (2 mM) and kept in the incubator for 15 h. They were washed again with PBS-1x and incubated with supplemented DMEM for 10 h. This double thymidine block procedure was used to induce a more synchronized S phase

blockade, and therefore enhance the number of dividing cells. Next, the cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. After three washes with phosphate-buffered saline (PBS-1x), the cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS-1x for 15 min at room temperature. They were then washed with PBS-1x, blocked with 3% BSA in PBS-1x for 1 h, and incubated with the following primary antibodies: rabbit anti-CENPH (1:100) (HPA036494, Prestige Antibodies, Sigma) and mouse anti-α-tubulin that recognizes the protein coded by *TUBA4A* (T6074, 1:1000) (Sigma), diluted in 3% BSA PBS-1x overnight at 4°C, followed by three washes with 3% BSA in PBS-1x, and then incubation with their respective secondary antibody: donkey anti-rabbit (1:500) (A10042, Invitrogen), donkey anti-mouse (1:500) (A10037, Invitrogen), diluted in 3% BSA PBS-1x for 1 h at room temperature. The slides were mounted using Vectashield hard-set mounting medium with 4–6 diamidino-2-phenylindol-2-HCl (DAPI) (Vector Laboratories) and examined using an Axioskop 2 plus epifluorescent microscope or a Zeiss LSM880 Laser Scanning Confocal. Photos were taken using the Carl Zeiss Zen 2012 SP1 software.

Results

The search for a rare recurrent and unique mechanism for RM

Given that RM is such a vast and heterogeneous entity, we decided to search for a rare recurrent and unique mechanism in hopes of better homogenizing this entity and facilitating gene identification. Our strategy to analyze the parental contribution to the various forms of fetal loss is illustrated in figure 2. We chose flow cytometry

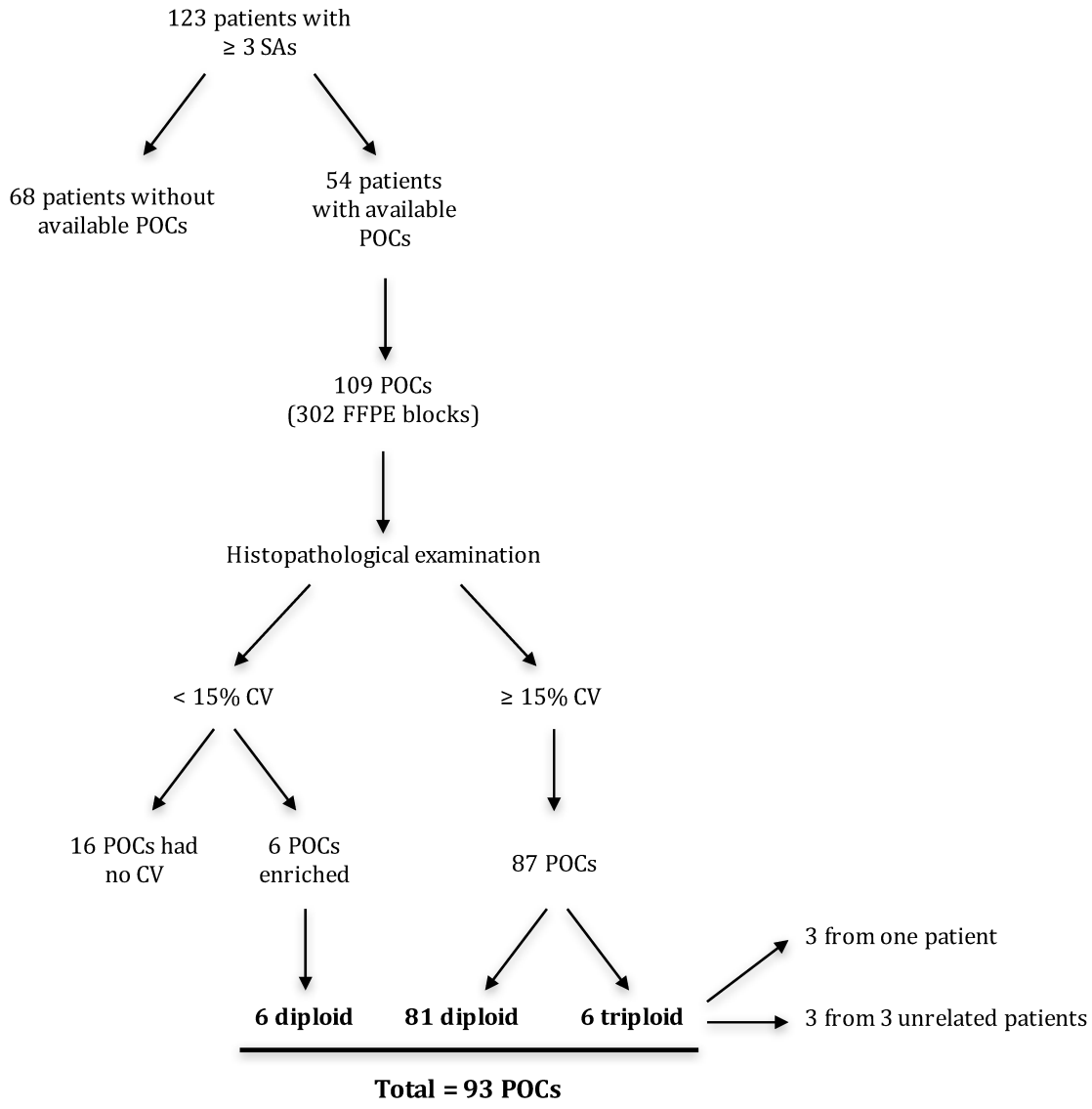

Figure 2. Strategy of analysis for the identification of aneuploid POCs.

as a first pass analysis because it is the fastest and cheapest method to determine the ploidy of POCs on archived formalin fixed paraffin embedded tissues.

Sensitivity of flow cytometry on FFPE tissues

After implementing flow cytometry on formalin-fixed paraffin embedded tissues in our laboratory, I first tested the sensitivity of the method and determined the minimum detectable level of CV in a paraffin block. This test involved mixing various amounts of CV previously shown to be triploid with normal diploid endometrial tissue taken from elective terminations of normal pregnancies. I found that my experimental protocol could detect an abnormal triploid peak if the paraffin block contains approximately 15% of CV based on morphological evaluation of its H&E slide using light microscopy.

Frequency and recurrence of triploidies in women with RM

We examined the H&E slides of 302 paraffin blocks from 109 POCs of 54 patients with RSAs under light microscopy. We had 87 paraffin blocks from 87 POCs that contained approximately at least 15% of CV. Twenty-two POCs had paraffin blocks with less than 15% of CV (figure 2). For six of these POCs, it was possible to enrich for the CV by taking from the sections only the regions that contained CV rather than taking the entire sections of the paraffin blocks. The remaining 16 POCs could not be analyzed because they had no or insufficient amounts of CV (figure 2). Thus, this analysis allowed us to reach a conclusion on 85% of the analyzed tissues.

Under these experimental conditions, we identified six POCs that are triploid (6.5%). Three of these are from three different patients, 554, 693, and 947, and the remaining three are from the same patient, 1138. Among the analyzed patients, 21 had at least two POCs included in the analysis and therefore, the frequency of recurrent triploidy in the 21 patients is one in 21 (5%). Two of these patients each had a single triploid conception along with a diploid one.

Flow cytometry was the initial method used to identify the triploidies (figure 3a). To confirm the identified triploidies with a second method, we performed FISH on tissue sections from the six triploid conceptions using centromeric probes from three chromosomes, X, Y, and 18. The FISH analysis confirmed the triploidy of the six POCs (Figure 3b-d) and revealed the absence of mosaicism based on the analysis of 100 nuclei from each POC.

Parental origin of triploidies in women with RM

To determine the parental origin of the six triploidies, we performed fluorescent microsatellite genotyping using the PowerPlex 16 HS System (Promega Corp., Madison, WI). This analysis demonstrated that all six triploidies are digynic (i.e. maternal origin of the extra set of chromosomes) based on the presence of two different maternal alleles (e.g. Figure 4a, marker FGA) or two doses of one maternal allele in the POCs (e.g. Figure 4a, marker D16S539) at four to nine markers from different chromosomes (Figure 4 and Table 2.3).

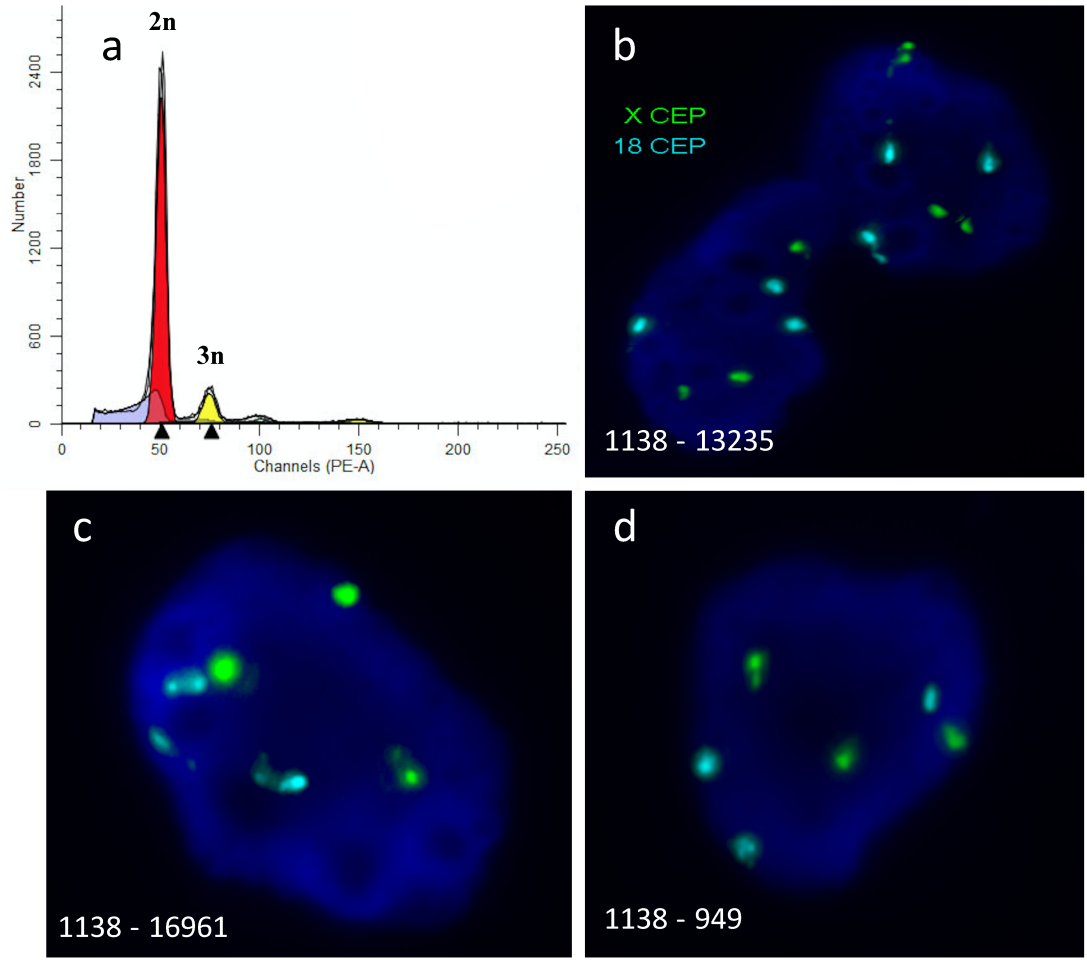

Figure 3. All three POCs of patient 1138 show three copies of chromosomes X and 18. A) Representative flow cytometry analysis revealing a triploid POC, b-d) tissue sections of three POCs from patient 1138 were hybridized with probes from the centromeres of chromosomes X and 18. The green color corresponds to the chromosome X probe and the cyan color to the chromosome 18 probe.

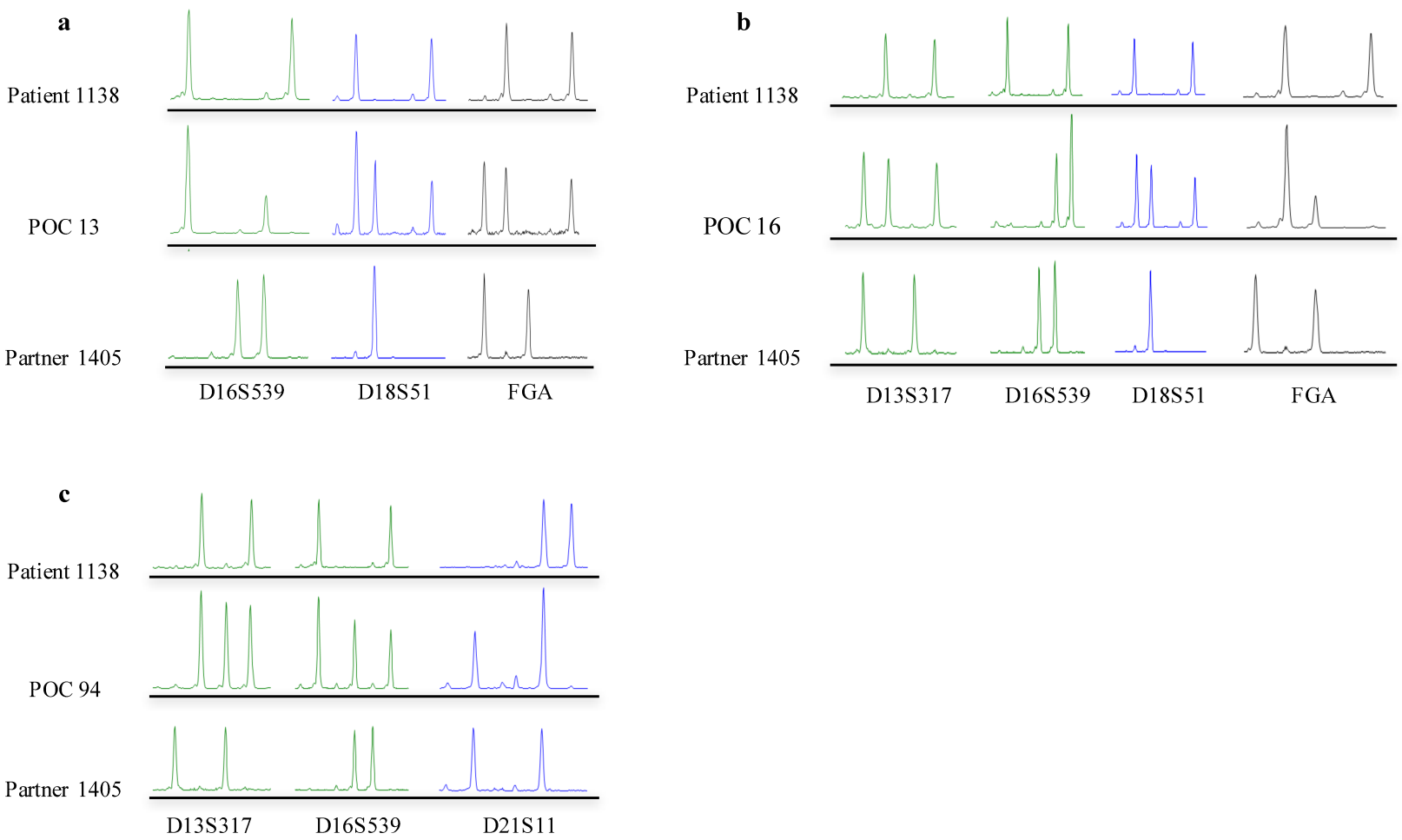

Figure 4. Multiplex genotyping results for the three POCs from patient 1138 reveal a digynic origin of the three triploid POCs. All markers show that the POCs received two alleles from the mother and one from her partner. The multiplex assay is quantitative and, depending on the heights of the peaks, allows us to determine if there are one or two doses of an allele. For example, in a), marker FGA shows two different maternal alleles in the POC and marker D16S539 shows two doses of one maternal allele in the POC.

Table 2.3. Genotyping with pericentromeric markers demonstrating reduction to homozygosity at informative markers in the mother

Marker	Start Position (Mb, hg38)	Centromere location (Mb, hg38)	Patient 554	POC 5854 of patient 554	Patient 693	POC 8314 of patient 693	Patient 947	POC 47439 of patient 947	Partner of patient 947
D7S1485	57.3	58.2-60.8	**200/208**	**200/208**/204 - NR	**212**	NA	**208**	**208**/204- UI	200/204
D6S402	62.3	58.6-59.8	**108/112**	**108/112**/116 - NR	**113/117**	**113**/123 - R	**113/115**	NA	104/115
D8S1115	42.9	44.0-45.9	**139/164**	139/164 - UI	**161**	NA	**161**	**161**/164 - UI	138/164
D18S869	22.5	15.8-20.6	**190**	NA	**185/189**	185/189 - UI	**182/186**	**182**/186 - UI	186
D11S1983	58.7	51.1-54.3	**236/244**	**236/244**/224 - NR	**226/230**	**226**/236 - R	**222/226**	**226**/242 - R	242
D12S2080	33.3	34.8-37.2	**182/186**	**182/186**/190 - NR	**182/186**	182/186 - UI	**185/189**	189 - R	185/189
D1S534	119.1	122.5-124.8	**198/200**	**198/200**/206 - NR	**202/206**	202/206 - UI	**202/206**	**206**/210 - R	204/210
D3S2462	96.4	91.6-93.7	**237/243**	**237/243**/241 - NR	**236/242**	236/242 - UI	**238/246**	**246**/248 - R	236/248
D4S3355	52.8	49.7-51.7	**127/135**	127/135 - UI	**134**	NA	**127/139**	**139**/160 - R	148/160
D14S122	20.9	16.4-18.2	**215/223**	**215/223**/219 - NR	**214/222**	222 - R	**196/214**	**214**/211 - R	203/211
D20S484	31.5	26.6-28.5	**177/189**	**177/189**/193 - NR	**189/197**	**197**/186 - R	**190/198**	**198**/186 - R	186/190

Marker	Start Position (Mb, hg38)	Centromere location (Mb, hg38)	Patient 1138	POC 949 of patient 1138	POC 13235 of patient 1138	POC 16961 of patient 1138	Partner of patient 1138
D7S1485	57.3	58.2-60.8	**204/212**	**204**/212 - UI	**204**/212 - UI	**204**/198 - R	198/212
D6S402	62.3	58.6-59.8	**109/113**	**109**/117 - R	**113**/117 - R	**113**/115 - R	115/117
D8S1115	42.9	44.0-45.9	**161**	NA	NA	NA	161
D18S869	22.5	15.8-20.6	**178/190**	178 - R	190 - R	178/190 - UI	178/190
D11S1983	58.7	51.1-54.3	**225**	NA	NA	NA	229/236
D12S2080	33.3	34.8-37.2	**182**	**182**/186 - UI	**182**/186 - UI	NA	186/194
D1S534	119.1	122.5-124.8	**208/212**	**208**/198 - R	**208**/204 - R	**208**/198 - R	198/204
D3S2462	96.4	91.6-93.7	**236/238**	**238**/236 - UI	**238**/236 - UI	**238**/236 - UI	236/242
D4S3355	52.8	49.7-51.7	**131/144**	**144**/135 - R	NA	**144**/135 - R	135
D14S122	20.9	16.4-18.2	**193/200**	**200**/219 - R	**200**/204 - R	**193**/204 - R	204/219
D20S484	31.5	26.6-28.5	**182/186**	**186**/194 - R	**186**/194 - R	**182**/186 - UI	186/194

Mb stands for mega bases; POC, for product of conception, NR, for non reduction; UI, for uninformative; R, for reduction; NA, for not available. Maternal alleles are bolded; double-dose alleles are underlined.

Mechanisms at the origin of the identified triploidies

To determine whether these triploid POCs were caused by errors affecting the proper completion of MI or MII, we genotyped their DNA with pericentromeric markers mapped at less than 5 Mb from the centromeres of several chromosomes (Table 2.2). Briefly, pericentromeric markers were used to minimize the chances of recombination and to determine whether maternal heterozygosity was maintained (non-reduction) or reduced to homozygosity (reduction) in the triploid digynic POC.[110] For example, in patient 554, at the pericentromeric marker D6S402, the mother has two alleles 108 and 112 that were both transmitted to the POC. The presence of the two maternal alleles in the POC indicates non-reduction (NR) and is consistent with an error that occurred before MI and prevented its normal occurrence and the separation of the two homologous chromosomes. In patient 693, at the same marker, D6S402, the patient has alleles 113 and 117, but only one of them, 113, was transmitted to the POC, which indicates reduction to homozygosity and thus MI must have taken place normally and the triploidy originated from failure of MII. If the triploidy resulted from failure of MI, one would expect non-reduction at all pericentromeric markers for which the mother is heterozygous, while if the triploidy resulted from failure of MII, one would expect reduction to homozygosity at markers that are heterozygous in the mother. Among the non-recurrent triploid digynic miscarriages, we found that one is due to failure of MI and two are due to failure of MII (Table 2.3). Interestingly, the three digynic POCs from patient 1138 were all found to be caused by failure of MII since all informative markers tested displayed reduction to homozygosity (Table 2.3).

Medical and family histories of patients with triploid conceptions

The six triploid digynic POCs identified in this study were from the following four patients:

1) Patient 554, who had a history of five SAs followed by six failed *in vitro* fertilization (IVF) attempts and two live births from a twin pregnancy after ovum donation. Her SAs and failed IVF occurred between the ages of 41 and 45 and her triploid conception was from a spontaneous conception at the age of 43. This patient had another POC that was diploid and the patient had been diagnosed with PCOS based on the presence of hirsutism, oligomenorrhea, and uncontrollable weight gain. She was given occasional doses of Provera to stimulate her periods.

2) Patient 693 had a history of one blighted ovum, four chemical pregnancies, one ectopic pregnancy, and one live birth after intra-uterine insemination. Her reproductive losses occurred between the ages of 29 and 33; only one was genotyped and found to be triploid. This triploid conception occurred at the age of 30. She was also diagnosed with PCOS based on oligomenorrhea and the presence of PCO by ultrasound. The patient was borderline underweight and was given metformin to regularize her menstrual cycles.

3) Patient 947 had six SAs between the ages of 34 and 36 and had been diagnosed with a borderline hypothyroidism and was under Synthroid treatment. Three of her POC were available for genotype analysis, two were found diploid and were separated by a triploid conception that occurred at the age of 36. She has one

sister who had a child with Tetralogy of Fallot, five SAs, thyroid carcinoma, and multiple uterine fibroids.

4) Patient 1138 had ten SAs between the ages of 27 and 31 that were followed by a live birth; all were from spontaneous conceptions. Three of her POCs were available for analysis and were found to be triploid. These triploid conceptions occurred at the ages of 27, 28, and 31. The patient was diagnosed with PCOS, based on ultrasonography and oligomenorrhea. She was treated with metformin to regularize her menstrual cycles. The recurrence of the same mechanism of triploidy in three of her conceptions suggested an underlying genetic defect. We therefore re-contacted the patient, reviewed her family history, and found that she has a significant family history of ovarian problems: two of her maternal aunts had ovarian cysts and one of them underwent left oophorectomy. She also has another aunt who suffered from infertility. On her paternal side, some of her relatives had one to two miscarriages. We note that on both of her parental sides, none of her relatives had a similar high number of miscarriages. This suggests that this patient most likely has a recessive monogenic defect inherited from both parents, or a digenic or polygenic defect caused by mutations or variants in at least two genes.

Therefore, among the four studied patients, patient 1138 was the best candidate for exome sequencing to search for her susceptibility gene(s) because of the recurrence of the same mechanism of triploidy and because of her family history. In addition to 1138, we included the three other patients with triploid conceptions in our exome sequencing, along with their available parents despite the

fact that these patients did not have recurrent triploidies as they could share the same mutations or responsible genes with patient 1138.

Analysis of whole-exome sequencing

Following exome sequencing, various filtering steps were applied to narrow down the number of variants. We considered recessive and dominant (from both parents) modes of transmission, the minor allele frequency (MAF) of the variants (in the 1000 genomes project, phase 3), and the scaled CADD score, which stands for the Combined Annotation Dependent Deletion score, a tool for scoring the deleteriousness of single nucleotide variants in the human genome (the higher the score, the more deleterious the variant). We excluded variants with a scaled CADD ≤ 10. Given the rarity of recurrent triploidy (about 1% of all conceptuses are triploid), we excluded variants with a global MAF (based on the 1000 genomes project, phase 3, www.1000genomes.org) that is greater than or equal to 0.005. We also considered variants that have an unknown MAF or CADD score. We excluded variants present in family members that do not have RM or PCO (when such members are available). A summary of the process for the different modes of inheritance is provided in figure 5.

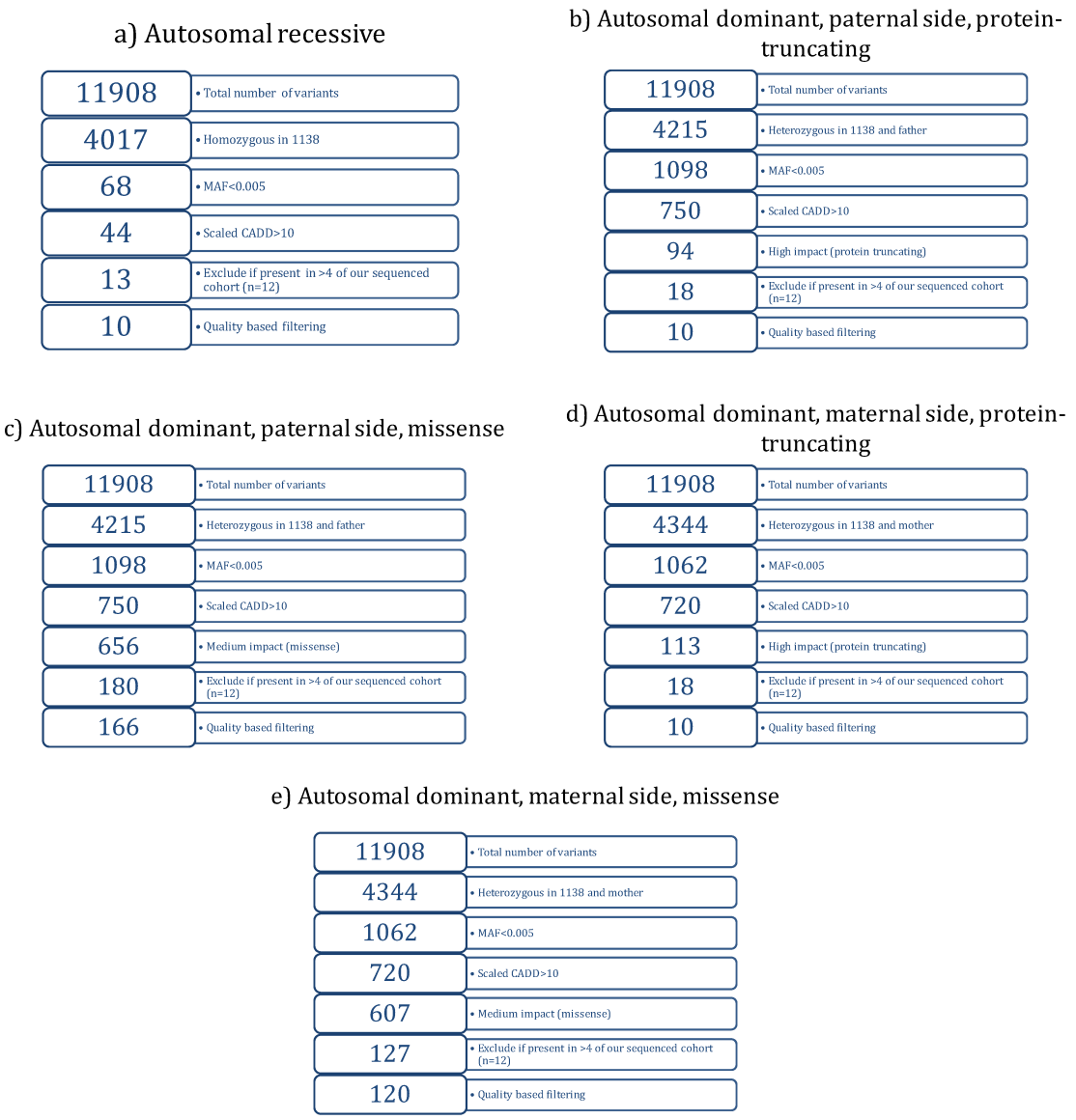

Figure 5. Summary for the filtration steps taken for each different mode of inheritance: a) autosomal recessive, b) autosomal dominant, paternal side, protein-truncating, c) autosomal dominant, paternal side, missense, d) Autosomal dominant, maternal side, protein-truncating, and e) autosomal dominant, maternal side, missense.

Selected variants in genes were then researched for possible roles in triploidy, such as genes known to be involved in cell cycle regulation, chromosome separation, meiosis, extrusion of the polar body, or associated with PCOS and prioritized them according to their conservation across species and functional impact on the proteins, as predicted by the scaled CADD and PolyPhen scores. In addition, we looked into the literature for genes reported to be linked to, or associated with, PCOS or miscarriage (Tables 1.5 and 2.4).

Results of whole exome sequencing

Under the recessive mode of transmission, we did not find any putative mutations either in homozygous or compound heterozygous state. Under the autosomal dominant mode, for variants inherited from the maternal side, we identified nine candidates, and our best candidate is Gene 1. In addition, we looked at the segregation of four genes that are involved in PCOS, even though these four genes did not meet all of our filtering criteria. The candidate genes' names will remain undisclosed for this thesis (Table 2.5).

Variants in the selected genes were validated by Sanger sequencing and their segregation was established in available and informative family members (figure 6). The variant in our best candidate, Gene 1, is novel (not present in any database), leads to protein truncation, and is present in the proband, the proband's mother, and one maternal aunt. The functional role of this gene suggests that it could explain the recurrent triploidy because it is a centromeric protein that is known to play a

Table 2.4. Putative PCOS susceptibility genes identified in GWAS

Gene	Full gene name	Reference
LHCGR	Luteinizing hormone/choriogonadotropin receptor	Chen et al., 2011
THADA	Armadillo repeat containing	Chen et al., 2012
DENND1A	DENN domain containing 1A	Chen et al., 2013
FSHR	Follicle stimulating hormone receptor	Shi et al., 2012
C9orf3	Chromosome 9 open reading frame 3	Shi et al., 2013
YAP1	Yes associated protein 1	Shi et al., 2014
RAB5B, SUOX	Member RAS oncogene family/sulfite oxidase	Shi et al., 2015
HMGA2	High mobility group AT-hook 2	Shi et al., 2016
TOX3	TOX high mobility group box family member 3	Shi et al., 2017
INSR	Insulin receptor	Shi et al., 2018
SUMO1P1	SUMO1 pseudogene 1	Shi et al., 2019
GATA4/NEIL2	GATA binding protein 4/nei like DNA glycosylase 2	Hayes et al., 2015
KCNA4/FSHB	Potassium voltage-gated channel subfamily A member 4/ follicle stimulating hormone beta subunit	Hayes et al., 2015
ERBB4	Erb-b2 receptor tyrosine kinase 4	Day et al., 2015
RAD50	RAD50 double strand break repair protein	Day et al., 2015
KRR1	Ssmall subunit processome component homolog	Day et al., 2015
KHDRBS3	KH RNA binding domain containing, signal transduction associated 3	Lee et al., 2015

Table adapted from Zhao et al., 2016

Table 2.5. Undisclosed candidate genes involved in meiosis, homologous recombination, ovarian insufficiency, and PCOS.

Gene	Full gene name	Mutation	MAF	Scaled CADD	Polyphen	SIFT
Autosomal recessive mode of inheritance						
None						
Autosomal dominant, inherited from the mother						
Gene 1	Centromeric protein	Splice_acceptor	Novel	17.6	-	-
Gene 2	Transcription factor	Non_syn_coding	0.0006	23.3	0.92	0.01
Gene 3	Nucleotide exchange factor	Non_syn_coding	Novel	25.6	0.988	0
Gene 4	Nuclease	Non_syn_coding	0.0016	17.5	0.536	0.13
Gene 5	Centrosomal protein	Non_syn_coding	0.0014	17.94	1	0
Gene 6	Dynamin	Non_syn_coding	Novel	22	-	-
Gene 7	Polymerase	Non_syn_coding	Novel	11.9	0.033	0.32
Gene 8	Binding protein	Non_syn_coding	0.0046	12.11	0.752	0.01
Gene 9	Sex hormone receptor	Non_syn_coding	0.001	14.35	0.079	0.02
Autosomal dominant, inherited from the father						
Gene 10	Exonuclease	Non_syn_coding	0.001	14.28	0.008	0.22
Selected good candidates based on their involvement in PCOS						
Gene 11	Binding protein	Non_syn_coding	Novel	7.38	-	-
Gene 12	Coiled-coil protein	Stop_gain	0.006	26.8	-	-
Gene 13	Sex hormone receptor	In_frame_codon_gain	Novel	-	-	-

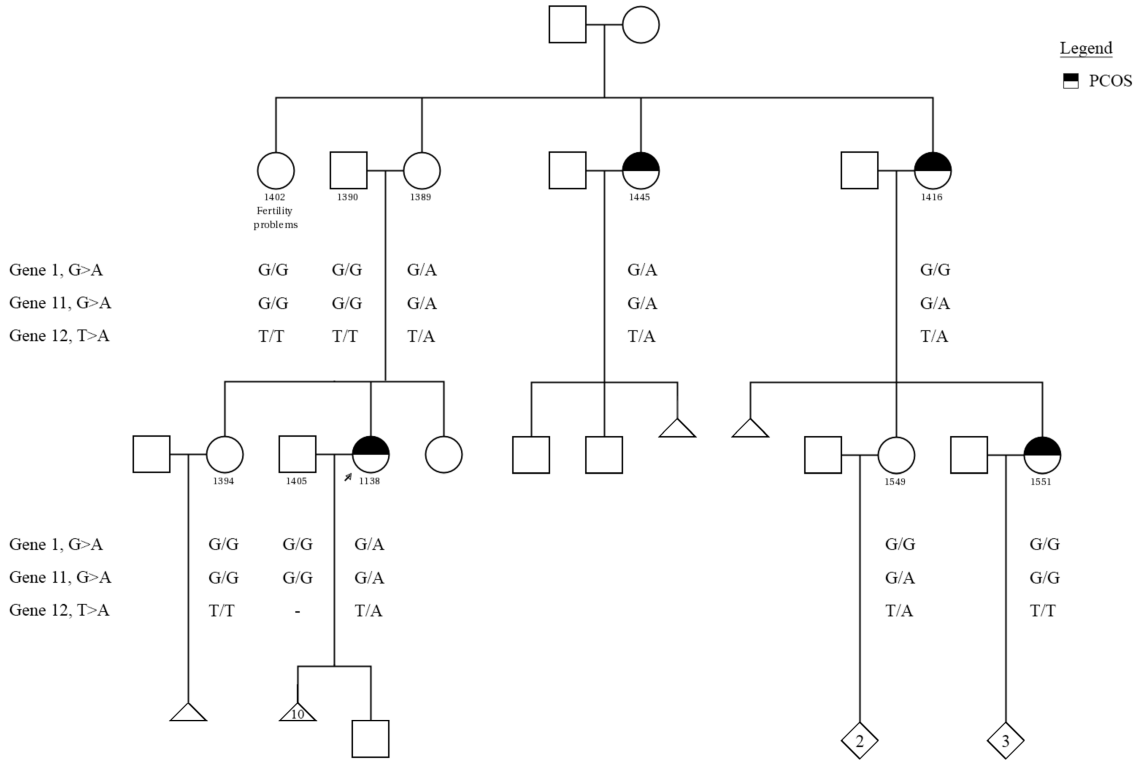

Figure 6. Pedigree structure of the family of patient 1138 and segregation of variants in three candidate genes. Gene 1 is functionally related to triploid miscarriages and Genes 11 and 12 with PCOS.

role in chromosome segregation and aneuploidies in human cancer cell lines, but has no known roles in meiosis. However, while the proband has ten miscarriages, the proband's mother did not have any miscarriages, and the aunt had only one.

Genes 11 and 12 are thought to play a role in PCOS. Looking at their segregation, the variants in these genes are found in two of the proband's affected aunts. However, they are also present in the mother and cousin, both unaffected, and absent in a cousin with PCOS. Therefore, based on their segregation in this family, they are unlikely to underlie the PCOS phenotype.

The rest of the candidate genes in table 2.5 are also interesting candidates and one of them is transmitted from the father. These variants will be validated and their segregation will be checked in the family after the submission of my thesis. They are involved in one of the following: homologous recombination, cytokinesis, the kinetochore, ovarian insufficiency, or PCOS.

Expression of candidate Gene 1 in human oocytes

To investigate whether Gene 1 may play a role in meiosis, we first checked its transcription in denuded human oocytes collected from an IVF clinic. We found that Gene 1 is expressed in denuded oocytes from all three stages: germinal vesicle, metaphase I, and metaphase II and replicated this finding in two independent pools of oocytes from each stage (figure 7). To check the localisation of this protein in human oocytes by immunofluorescence, I first tested its antibody on HeLa cells and observed a signal at the kinetochore of dividing cells (figure 8, a-d), but I have not yet been able to obtain a signal with this antibody on human oocytes, in the two

experiments I performed so far. I will repeat the experiment after my thesis submission as soon as I obtain these scarce materials, human oocytes.

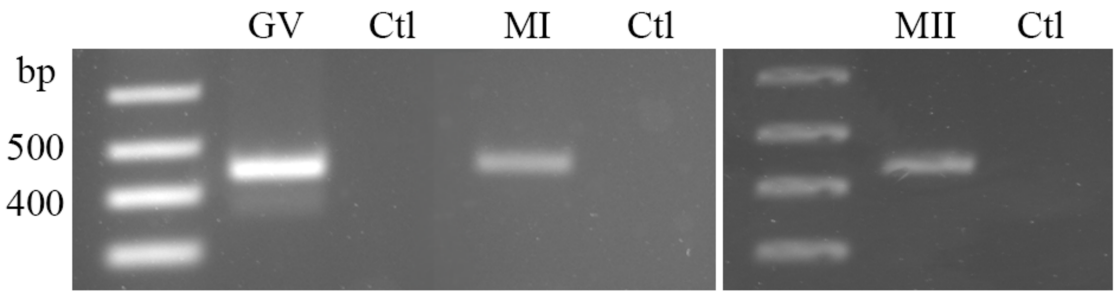

Figure 7. Gene 1 expression in denuded human oocytes. The GV stage is based on cDNA that is from two denuded GV oocytes; MI is from four denuded MI oocytes; and MII is from three denuded MII oocytes. The lysis buffer used to lyse the denuded oocytes was used as a control for the RT-PCR, and is shown for each stage. An additional negative water control, not displayed in the figure, was performed to control for the PCR reaction. Ladder used is the 1 Kb Plus DNA Ladder from Invitrogen.

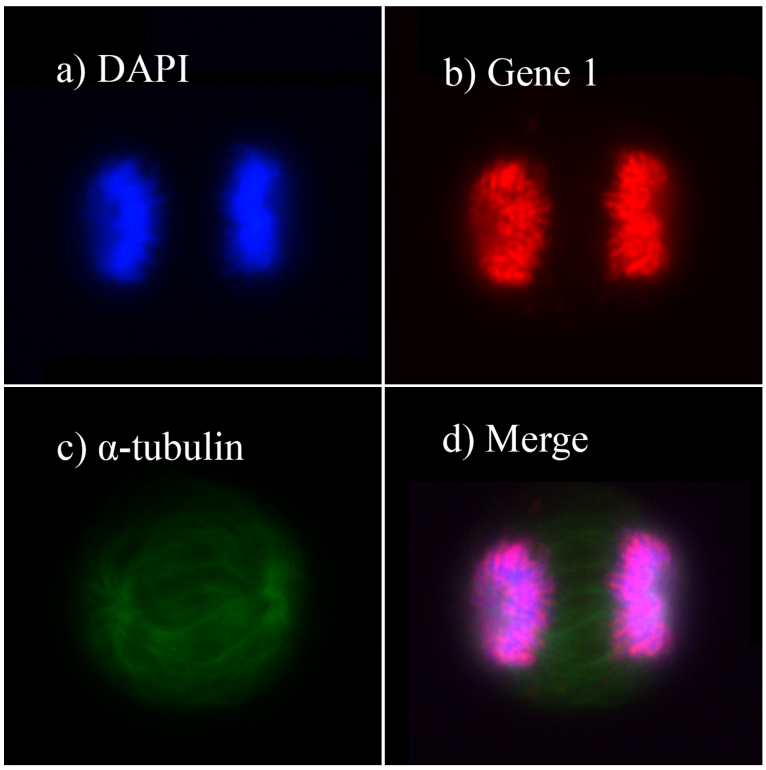

Figure 8. Localization of Gene 1 to the kinetochores during mitosis, in the late anaphase stage. HeLa cells were stained with a) DAPI, b) Gene 1, and c) α-tubulin; d) is a merge. Gene 1 overlaps with DAPI, as expected. The α-tubulin shows that the chromosomes are moving towards opposite cell poles.

Chapter 3: Conclusions and future directions

Flow cytometry is a relatively fast and inexpensive method to determine the ploidy of POCs. It is advantageous in that it does not require fresh tissues and can be performed retrospectively on FFPE tissues that are systematically prepared for all arrested pregnancies in Western countries as part of patient care. In this study, we used flow cytometry to assess the ploidy of 93 POCs from patients with ≥3 miscarriages. This analysis allowed us to reach a conclusion on the ploidy of 85% of the FFPE POCs and identify six cases of triploidies (6.5%). We therefore believe that flow cytometry on FFPE POCs is an important laboratory test that needs to be added to the evaluation of patients with RM since it provides the physician with evidence of abnormal meiosis and therefore an oocyte defect that can help in the guidance of the patients to appropriate ART services. Triploidy due to the fertilization with two spermatozoids usually leads to a partial hydatidiform mole.

We then confirmed the six triploidies by FISH and found that all are of maternal origin, one due to failure of MI and five due to failure of MII. The three triploid POCs from the same patient, 1138, all resulted from failure of maternal MII. Triploidy is one of the most common chromosomal aneuploidies at conception and occurs in 1-2% of clinically recognized conceptions.[116] In first trimester miscarriages, the frequency of triploidy is approximately 6.3% to 9.3% depending on patients' ascertainment criteria (non-recurrent miscarriages, patients with ≥2 miscarriages, or ≥3 miscarriages) and methodologies used to identify the triploidies.[68, 73, 117-122] In our study, the frequency of triploidy is 6.4% out of the analyzed POC, and is in agreement with data from previous studies.[68, 73, 117-122]

Our study is the first to assess the ploidy of POCs of patients with RM that are subdivided based on their clinical and laboratory evaluations, and to identify that maternal triploidy is not equally distributed among the various categories of RM, but seems to be higher in patients in the category "clinically unexplained." Interestingly within this category, five of the six triploid conceptions came from patients who had PCOS (p-value=0.008). However, given our small sample size, this finding warrants further investigation. Triploidies of maternal origin are due to female meiotic errors that occur before or after fertilization. Among the six categories of abnormalities in which we classified our patients with RM, abnormalities affecting pelvic anatomy, thrombophilia, and the immune system are not expected to affect female meiosis and lead to triploidies but rather to affect maternal and/or fetal vasculature and embryonic development after implantation. Consequently, one would expect RM from such patients to be diploid, which is in agreement with our data on their POCs. However, we were surprised to see that five of the six identified triploidies occurred in patients with PCOS. This suggests that triploid conceptions in patients with ≥3 RM may not be due to random meiotic errors but reflect an underlying ovarian problem. Among the analyzed POCs from patients with PCOS, triploid conceptions occurred in 28% (5 out of 18 POCs) and in three out of nine patients (33%), which is higher than the frequency of triploidies in women with sporadic miscarriages (6.4%). In addition, one of the five patients with PCOS had recurrent triploidies due to the same mechanism, and has a strong family history of polycystic ovaries (PCO). Altogether our data indicate an association between PCO and failure of female meiosis that would need to be validated in future studies on other cohorts.

To date, six cases of recurrent triploidies of maternal origin have been reported,[123-128] but a demonstration of the maternal origin in at least two POCs from the same patient has only been shown in two studies.[125, 128] In the latter paper the authors neatly demonstrated the recurrence of maternal triploidy due to failure of maternal MII in six conceptions from the same patient.[128] Similarly in our patient 1138, the recurrence of the same mechanism of triploidy in three conceptions points to a strong genetic predisposition underlying the three triploidies.

From the exome sequencing data, Gene 1 is currently our best candidate. It is functionally highly relevant and the variant is deleterious and pathogenic. However, the segregation is not optimal. It is possible that there is incomplete penetrance to explain why the two individuals who have the variant do not exhibit the phenotype. As for Genes 11 and 12 for the PCOS phenotype, again the segregation does not support a causative role for these genes and this family alone is not sufficient to investigate potential association between these variants and PCOS. Therefore, this case remains unsolved. It could be that the causative gene is among the candidate genes that we have not yet tested and that the traits (triploidy and/or PCOS) are multifactorial, resulting from digenic or polygenic inheritance.

Though we could not yet come to a conclusion regarding the causative gene(s) for these phenotypes in this familial case, we found a patient with recurrent triploidy due to the same repeated mechanism, and we have a number of potential candidate genes. We believe that such rare cases are great opportunities for research and a means to increase our knowledge and understanding of the vast and incredibly heterogeneous entity of recurrent miscarriage. Within the entity of RM, in addition to the challenge of finding cases with a unique and recurrent mechanism,

there's the bigger challenge of finding another patient with the same genetic defect, given the genetic heterogeneity of this entity. As such, working with an animal model to study candidate genes and their functional impact may be the best approach for future research.

Future directions

After the submission of this thesis I will determine the segregation of the remaining interesting candidate genes in the family of patient 1138. After that, one direction to take with this work is to perform targeted exome sequencing on our top 5-8 genes in our cohort of 37 patients with RM from category 5) "Clinically unexplained" (some of whom have PCOS) (Table 2.1).

Further, we have a number of new POCs from patients with RM. The miscarriages from these cases need to be studied, as was done before – flow cytometry to determine ploidy, multiplex genotyping for the triploid cases to identify the parental origin, and finally simplex genotyping using pericentromeric markers to determine whether the triploidy is due to an error in meiosis I or meiosis II. The goal would be to identify new patients with PCOS and triploidy of maternal origin to replicate the association we found.

We are also planning to contact two other investigators who published the only existing reports about two unrelated patients with recurrent triploid conceptions of maternal origin[125, 128] and propose to collaborate with them by sharing DNA or exome data with us. This will increase our chances of identifying a

causative gene for recurrent triploidies by the identification of a second patient with the same or different mutations in the same gene.

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