Genetic diagnosis of subfertility – the impact of meiosis and maternal effects.

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Journal of Medical Genetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>jmedgenet-2018-105513.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Review</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>24-Dec-2018</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Gheldof, Alexander; Universitair Ziekenhuis Brussel, Centre for Medical Genetics Mackay, Deborah; University of Southampton, Faculty of Medicine Cheong, Ying; University Hospital Southampton NHS Foundation Trust, Princess Anne Hospital, Division of Women and Newborn Verpoest, Willem; Universitair Ziekenhuis Brussel (UZ Brussel), Vrije Universiteit Brussel (VUB), Centre for Reproductive Medicine</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Clinical genetics, Diagnostics, Genetics, Genetic screening/counselling, Obstetrics and Gynaecology</td>
</tr>
</tbody>
</table>
Genetic diagnosis of subfertility – the impact of meiosis and maternal effects.

Alexander Gheldof¹,², Deborah Mackay³, Ying Cheong⁴, Willem Verpoest⁵,²

¹ Center for Medical Genetics, Universitair Ziekenhuis Brussel, Brussels, Belgium
² Reproduction and Genetics Department, Vrije Universiteit Brussel, Brussels, Belgium
³ Faculty of Medicine, University of Southampton, Southampton University Hospital, Southampton, UK
⁴ University Hospital Southampton NHS Foundation Trust, Princess Anne Hospital, Division of Women and Newborn, Southampton, UK
⁵ Center for Reproductive Medicine, Universitair Ziekenhuis Brussel, Brussels, Belgium

Corresponding Author:
Alexander Gheldof,
Center of Medical Genetics, Universitair Ziekenhuis Brussel
Laarbeeklaan 101, 1090 Brussels, Belgium
alexander.gheldof@uzbrussel.be

Word Count: 6628

Keywords: Premature ovarian insufficiency, meiosis, maternal effect genes, gene panel testing,
Contributorship statement
AG wrote the main text, DM and YC wrote the sections concerning maternal effect genes and critically read the manuscript, WV conceptualized the study and critically reviewed the manuscript.

Funding:
This project was funded by a Willy Gepts Scholarship.

Competing interests
None
Abstract
During reproductive age, approximately one in seven couples are confronted with fertility problems. While the etiology is diverse, including infections, metabolic diseases, hormonal imbalances and iatrogenic effects, it is becoming increasingly clear that genetic factors have a significant contribution. Due to the complex nature of infertility which often hints at a multifactorial cause, the search for potentially causal gene mutations in idiopathic infertile couples has remained difficult. Idiopathic infertility patients with a suspicion of an underlying genetic cause can be expected to have mutations in genes which do not readily affect general health, but are only essential in certain processes connected to fertility. In this review, we specifically focus on genes involved in meiosis, a process which plays a pivotal role in fertility, and genes critical for maternal effect processes. We give an overview of genes which have been linked to infertility, as well as genes which are good candidates. Finally, we propose a phenotypic range in which we expect an optimal diagnostic yield of a meiotic/maternal effect gene panel.

Acronyms: RPL: recurrent pregnancy loss; SPGF: spermatogenic failure; POF: premature ovarian failure; POI: primary ovarian insufficiency; MI: meiosis I, MII: meiosis II.
**Background**

It is estimated that 10-15% of couples are affected by infertility during reproductive age, with equal distribution of subfertility between males and females[1]. However, a significant proportion of couples are unsuccessful despite having healthy reproductive age, no detectable physical, endocrine or immune problems, apparently adequate quantity and quality of gametes, and no apparent technical laboratory issues affecting the ART procedures. For example, 50-80% of cases diagnosed with primary ovarian insufficiency (POI) remain idiopathic [2, 3]; likewise, in 80% of men with non-obstructive azoospermia the cause remains unknown [4]. For such individuals there are currently limited options for intervention to optimize fertility. When confronted with idiopathic infertility patients, an important first test which is often used by fertility centers, is karyotyping. In a cohort study of 1663 azoospermic men, 14% of the tested individuals had chromosomal abnormalities, stressing the importance of karyotyping in fertility patients as a first tier test [5]. Patients with a normal karyotype and with exclusion of other causes may however be warranted to undergo genetic analysis.

In a clinical setting, one of the routes which can be followed to accomplish this is diagnostic gene panel sequencing. In humans to date, only a limited number of genetic changes have been found, affecting fertility in small numbers of cases [4, 6]. These findings hint at a multifactorial genetic origin and/or environmental influences [7]. In this scenario, the setup of genetic studies for infertility faces the risk of being underpowered, not only because of an insufficient amount of samples, but also due to difficulties in clearly delineating the clinical pathophysiology. Therefore, to potentially increase the diagnostic yield of gene panels, both the patient phenotype and the disease spectrum of the investigated genes should be matched as well as possible. For example, when investigating the genetic causes of subfertility of individuals with no other overt health problems, and without other physical, environmental, endocrinological or structural problems, one of the potential causes could be found in the process of meiosis, an absolute prerequisite for both male and female gamete formation. In addition to this, defects in maternal effect processes could be suspected as well. Furthermore, errors in both processes are expected to lead to few secondary findings besides reproductive issues.

We here suggest that during IVF treatment, errors in meiotic and maternal effect genes, can, in absence of an overt male factor, lead to a reduced fertilization rate and an impaired early embryonic development. Meiotic defects have furthermore been described to be implicated in premature ovarian insufficiency (POI) as well [8, 9]. However, the genetics of POI is broad, while in this review, the emphasis is put on meiotic and maternal effect genes with a potential clinical implication in infertility. Since genetic and functional evidence from humans is limited, our study will be mainly based on reports from animal models. Most particularly, research in mice has explored many reproductive processes and identified critical factors, and animal studies are cited when relevant.
with the understanding that species differences limit the power of extrapolation to humans.

**Meiosis**

Meiosis is an essential process of gamete formation and its genetic disruptions are likely to have a considerable impact on fertility. Expression of meiosis genes is implicated in considerations including ovarian reserve, ovarian response, and oocyte maturation and activation. Meiosis gene mutations may therefore lead to a number of clinical pathologies such as premature ovarian insufficiency (POI), insufficient oocyte maturation and low fertilisation rate.

Several distinct steps are necessary for meiotic completion, including the formation of double-strand breaks, chromosome synapsis, homologous recombination, separation of homologous chromosomes during MI and separation of sister chromatids during MII. Since the spatiotemporal regulation of meiosis is also dependent on somatic cells in humans, namely the granulosa cells in women and Sertoli cells in men, genes involved in the crosstalk between the somatic and the germline compartment are also relevant to meiotic success.

Below, we describe the molecular subprocesses of meiosis and as such define a collection of genes warranting inclusion in a diagnostic gene panel for idiopathic infertility. This will comprise both genes that have already been described in an idiopathic fertility setting, as well as unreported genes that have a high potential to lead to meiotic errors when disturbed (Fig. 1).

- The synaptonemal complex: basis for chromosome pairing, synapsis and recombination

An essential premise for meiosis to take place is the correct alignment of homologous chromosomes (pairing) during its initial stages. A crucial mediator for this process is the synaptonemal complex (SC), a multiprotein structure which is assembled during meiotic prophase I and which is essential for synapsis, meiotic crossover[10] and correct segregation of homologous chromosomes during anaphase in the first meiotic division [11]. Given the pivotal role of the SC in meiosis, mutations in SC would be expected to give rise to fertility problems.

The SYCP3 protein is, together with SYCP2, one of the main components of the lateral elements of the SC and is essential for chromosome loading on the SC [12]. Mutations in SYCP3 have been shown in men with non obstructive azoospermia [13]. Examination of testicular biopsies revealed that the most mature spermatogenic cells were early spermatocytes, indicating a meiotic arrest, whereas SYCP3 mutations in women do not seem to lead to a meiotic arrest, but result in recurrent pregnancy loss, probably due to the presence of aneuploidies [14]. This sexual dimorphism is speculated to arise from greater stringency of the pachytene checkpoint in men than in women [10]. To date, no mutations have been found in SYCP2, but mouse Sycp2 mutants show a phenotype reminiscent of human SYCP3 mutations, including the sexual dimorphism [15]. Females
lacking the SYCP2-like gene product SYC2PL undergo accelerated reproductive ageing [16].

Mutations in the SC component SYCE1 have been reported in cases of human infertility [17]. SYCE1 is a component of the central element of the SC. Both male and female Syce1-mutant mice are infertile and are characterized by an arrest in prophase I [18]. Reports of human SYCE1 variants identify azoospermia in affected males and females affected by POF [17, 19]. In mice, Meiob and Spata22 both form discrete foci on meiotic chromosomes; the absence of either causes loss of the other from these meiotic foci, along with failure of meiotic synapsis. Although Meiob ablation is associated with both male and female infertility in mice, in humans MEIOB mutation has been associated only with male azoospermia [20, 21]. Murine ablation of Spata22 is also associated with male and female infertility through failure of synapsis[22].

- Double strand break formation

Precise alignment of the homologous chromosomes allows the initiation of the next meiotic process, recombination or crossing over (Fig. 1). Crossover occurs at one or multiple sites along the length of each chromosomal arms, resulting in the formation of chiasmata, and these chiasmata are essential to maintain chromosome cohesion during meiosis. Reduced recombination or incorrect placement of chiasmata is associated with increased incidence of aneuploidy [23–25]. Paucity of chiasmata is most likely to lead to aneuploidy in the smallest chromosomes where the overall length of the chromosome is smallest, for example, chromosome 21; but there is evidence that the genome-wide frequency of crossover may have some genetic basis. In families where one offspring has Trisomy 21, genome-wide analysis indicates that the frequency of crossovers is reduced, not only in the individual affected by Trisomy 21 but also in siblings [26]; and this crossover frequency may be partly accounted for by variation in the recombination factor PRDM9 [27].

Interestingly, the helicase-homologous protein HFM1, expressed in male and female germ tissues, appears to be required for formation or resolution of crossovers; in mice lacking this gene product, early steps in crossover are normal, but then most crossovers are eliminated and the majority of germ cells undergo apoptosis [28]. Human HFM1 variants have been identified in women affected by POF [29]. MCM8 and MCM9 are more widely expressed in somatic tissues, and their ablation results in accumulation of DNA damage in response to replication stress, but nonetheless the key phenotype of mice lacking these proteins is infertility, apparently due to errors in HR [30]. Variants in MCM8 have been identified in women affected by POF [31, 32].

Meiotic crossover requires the creation of double-strand breaks (DSB) in individual chromosomes, and subsequent recombination between chromosome homologues. Meiotic DSB generation requires the highly conserved SPO11 topoisomerase-like protein (Fig. 1). In human, heterozygous SPO11 mutations have been shown in men with azoospermia [33]. In mouse models entirely lacking Spo11, spermatogenesis arrested before the pachytene stage, while oocytes arrested in

https://mc.manuscriptcentral.com/jmedgenet
prophase I [34, 35]. *SPO11-/-* preleptotene spermatocytes lacked homologous pairing, independent of the SPO11 DSB catalytic activity [36]. However, in a hypomorphic mouse model expressing 60% normal levels of *Spo11*, spermatocyte development was normal [37], and *Spo11 +/-* male mice showed no reduction in fertility compared to wild type animals [38].

Genetic defects in the regulatory machinery of SPO11 could also contribute to a fertility phenotype. Studies in yeast have delineated distinct mechanisms for SPO11 regulation in meiosis, either through intrinsic control of SPO11 dimerisation and nuclear retention, or through regulation of its interaction with DNA recombination hotspots. For instance, Rec102, Rec104 and Ski8 are required for SPO11 dimerization, DNA binding and nuclear retention in yeast [39–41]. On the other hand, the SPO11 accessory proteins REC11, Mer2 and Mei4 form a complex which is essential for the DNA binding and guiding of SPO11 to DSB cleavage sites [42]. *Mei4 -/-* male mice are unable to initiate DSB formation in meiosis, resulting in synaptic defects and arrest of spermatogenesis [42]. Mutations in homologous *SPO11*-associated genes have not yet been described in humans. In mice, an additional factor which has been shown to be necessary for DSB formation/maintenance is *Hormad1*. Knock-out mice meiocytes show a strong reduction in single stranded DSB ends, as is evidenced by the diminished presence of Dmc1/Rad51 foci [43]. As both Hormad1 and its close paralog Hormad2 associate with the axis of unsynapsed chromosomes and have been hypothesized as inhibitors of interstrand DNA repair, thus favourizing interhomologous driven repair, chromosome synapsis is disrupted as well in the *Hormad1/2 +/-* models [44, 45]. Upon synapsis, Hormad1/2 dissociate from the chromosomal axis, a process which is facilitated by Trip13. Trip13 +/- mice oocytes show full chromosome synapsis, but are unable to repair the Spo11 mediated DSBs, further supporting the role of Hormad1/2 in interhomologous repair [46]. Failure of DSB repair leads to Chk2 dependent oocyte clearance. Interestingly, while testes of *Hormad1 -/-* mice show progressive atrophy, ovarian development does not seem to be affected [47]. However, embryos of *Hormad1 -/-* females do not proceed further than the blastocyst stage.

- Homologous recombination

Creation of meiotic DSB is followed by homologous recombination, which is driven by cellular DNA repair machinery that is shared between germline and somatic cells (Fig. 1). DSB repair is initiated by the Mre11-Rad50-Nbs1 complex which attracts both the ATM and ATR kinases to the DSB sites, and which in their turn phosphorylate histone H2AFX that acts as a beacon to attract novel repair associated proteins [48]. In addition, ATM phosphorylates multiple DNA damage repair associated factors including CHK2, BRCA1/2 and P53, which subsequently orchestrate crucial cell cycle checkpoints and the potential decision to drive the cell towards apoptosis if DSB repair is unsuccessful [49]. Repair by homologous recombination (HR) is mediated by DMC1 and Rad51 proteins which form a nucleosome complex around the single strand overhangs of DSBs. Rad51 is an essential facilitator for DMC1 mediated inter-homologous strand invasion [50]. Interaction of
the DMC1/Rad51 complex with the strand invasion structure is furthermore enhanced by the Hop2-Mnd complex [51]. Spermatocytes or oocytes with unrepaired DSBs are expected to be eliminated due to apoptosis or undergo induced senescence. Dmc1-/- mice ovaries are devoid of follicles, while depletion of Chk2 can rescue the phenotype by preventing Chk2 dependent Trp53 (p53 in humans) activation and subsequent apoptosis [52]. In humans, meiotic DSB repair is furthermore facilitated by the MSH4-MSH5 heterodimer, which specifically associates with Holliday junctions thereby stabilizing the DSB intermediates [53]. MSH4/5 proteins are members of the MutS homologs which are mainly implicated in mismatch repair (MMR). While MSH2, 3 and 6 are implicated in mitotic MMR, MSH4/5 are specifically active during meiosis. Interestingly, MSH5 is also expressed in granulosa cells [54]. Mutations in both MSH4 and MSH5 have been detected in POI families [54, 55].

DNA repair deficient mice often results in early lethality, as has been demonstrated for Rad51, PalB2, Brca1 and Brca2 knockout mice [56]. Human mutations in DSB repair genes including ATM, ATR, CHEK2, RAD51 and BRCA1/2 are associated with morbid phenotypes including cancer predispositions, and to date, no clear link has been demonstrated between hypomorphic variants in these genes and an infertility phenotype. It is not clear at present whether they warrant inclusion in an infertility gene panel.

The specific case of BRCA1 and BRCA2 deserves further mention. Both proteins are involved in DSB repair and resolution of HR, and women carrying inactivating mutations are at elevated risk of cancer. A recent metastudy of carriers of BRCA1/2 variants did not reveal significant subfertility compared to a normal control population [57]. However, BRCA2+/- mice show a significant reduction in germline cells [58]. Spermatocytes do not progress further then early prophase I, while oocytes have been shown to progress through prophase, albeit with the presence of nuclear abnormalities. Likewise, BRCA1+/- mice are subfertile, characterized by an increase in oocyte apoptosis after hormonal stimulation and smaller litter sizes [59]. Notwithstanding these observations, the association of BRCA variation with cancer susceptibility mandates caution in including these genes in a fertility gene panel.

- Meiosis: cohesin is key

Upon completion of homologous recombination, the first meiotic division (MI) is initiated. To keep the sister chromatids together until separation in MII, spatiotemporal regulation of the cohesin complex is necessary. While the cohesin complex is located along the whole length of the sister chromatids during synapsis and homologous recombination, cohesin is depleted from the arms of sister chromatids after MI but from centromeres only in MII [60] (Fig. 1). Protection of centromeric cohesin prevents premature separation of the sister chromatids at MI which could result from the mechanical pull of the kinetochores, but would lead to aneuploidy. In humans, the cohesin complex consists of Smc1, Smc3, Rec8 and a STAG1-3/Scc3 subunit [61]. After homologous recombination,
phosphorylation of cohesin subunits (in particular Rec8) along the length of the sister chromatids permits separase cleavage of Rec8, breakdown of the cohesin complex and separation of chromatid arms [60]. At the centromeres, cohesin association with the shugoshin-PP2A phosphatase complex blocks phosphorylation and prevents premature separase-induced cleavage. When cells enter MII, the shugoshin-PP2A complex is antagonized by the SET protein, allowing Rec8 cleavage by separase and separation of the sister chromatids [62].

Meiotic segregation errors (leading to aneuploidy) increase in frequency with age, because of the incremental depletion of cohesin and Sgo2 [63]. Both male and female mice lacking Sgo2 are infertile, but in humans SGO2 mutation has been reported only once to date [64]. In mice, Sgo2 is furthermore stabilized by Meikin and consequently, oocytes of Meikin -/- females display a disrupted anaphase II due to premature separation of the sister chromatids [65]. Furthermore, human homozygous mutations in STAG3 are associated with POF [66]. This has been mimicked in Stag3 -/- mice where further investigation showed a meiotic arrest at prophase I, leading to oocyte depletion. Moreover, mice which have a knock-out for Rec8, the phosphoprotein acting as a switch for separase degradation, are born in a submendelian frequency and are sterile [67]. However, since other cohesin subunits are essential for both mitosis and meiosis, mutations in these result in congenital morbidities rather than reproductive disorders; for example, SMC1A mutations cause Cornelia de Lange syndrome, an X-linked dominant disorder characterized by growth retardation, developmental delay and often microcephaly [68, 69]. It remains possible that hypomorphic variants in cohesin complex components and regulators may produce reproductive effects, warranting their inclusion in a diagnostic gene panel for fertility.

• Failure of completing MI or MII: meiotic arrest

The impossibility of an oocyte to complete MI or MII will, in case the oocyte pool is not fully cleared in the ovaries, likely result in fertilization failure even when ICSI is applied and this can in theory be caused by mutations in any of the genes described above. However, during recent years, multiple novel genes have been described as being essential for meiotic progression. Although most work has been performed in mice and Xenopus, it can be expected that similar effects can be seen in humans in the homologous genes. Mutations in PATL2 (shown in humans, mice and Xenopus), Lfng (shown in mice), Prkar2b (shown in mice), Cks1, Cks2, Mos (shown in Xenopus and mice) and Smc1b all have been shown to lead to failure to proceed through meiosis [65, 70–74]. The processes these genes are involved in, are diverse. For instance, oocytes of Cks2 null mice fail to proceed after prophase I and while the same holds true for Cks1 null mice, the Cks2 null oocytes can be rescued by microinjection of Cks1 mRNA [70, 75]. Both CKS1 and CKS2 bind to CDK1 and CDK2 (cyclin dependent kinases 1 and 2, respectively) complexes thereby modulating the cell cycle [76]. Interestingly, in Xenopus, it has been demonstrated that the CKS1 homolog strongly
enhances phosphorylation of the downstream CDK target Myt1, by which meiosis I entry is enabled [77, 78]. Furthermore, entry into MI in \textit{Xenopus} requires Mos activation, which, in turn phosphorylates Myt1 [79]. Mos, which is an upstream activator of the MAPK pathway (Mitogen Activating Protein Kinase), is also implicated in maintaining the oocyte MII arrest (shown in mice and in \textit{Xenopus}) by indirectly phosphorylating EMI2, an inhibitor of the anaphase promoting complex (APC) [80]. A complementary mechanism by which MI is arrested prior to the estrous cycle is through cAMP mediated phosphorylation of PKA (cAMP-dependent protein kinase) which activates the kinase Wee2 (or Wee1b), which in turn will phosphorylate CDK1, allowing the maintenance of prophase arrest [81]. Intriguingly, when the oocyte has progressed to MII, Wee2 is also necessary for final MII exit by phosphorylation of its target Cdc2. In mice oocytes, inhibition of Wee2 results in failure of pronucleus formation and consequently to the impossibility of fertilization [82]. Likewise, in humans, it has recently been shown that homozygous \textit{WEE2} mutations result in oocyte fertilization failure. Injection of \textit{WEE2} mRNA could compensate for the mutations and effectively resulted in fertilization [83].

In contrast to cell cycle modulation, the Lfng protein is a regulator of Notch signaling by posttranslational modification of the N-acetylglucosamine content of the Notch receptor, resulting in alteration of its ligand binding capacity [73]. While \textit{Lfng} \textit{-/-} mice are not born at mendelian ratio’s, the surviving female mice are subfertile and are characterized by significantly reduced \textit{in vitro} fertilization rate as the consequence of failure to proceed to meiotic metaphase II [73]. Interestingly, chemical inhibition of the Notch pathway in isolated mouse ovaries results in a marked downregulation of \textit{Lhx8}, \textit{Figla}, \textit{Sohlh2} and \textit{Nobox} mRNA expression [84]. In humans, mutations in \textit{LHX8}, \textit{FIGLA}, \textit{Sohlh2} and \textit{NOBOX} have been demonstrated to lead to POI, thus providing a link between Notch signaling, meiotic arrest and POI [85]. Furthermore, besides the Notch pathway, cAMP-dependent protein kinase (PKA) signaling is crucial as well in meiotic progression. For instance, during oocyte maturation, the PKA regulator, PRKAR2b is highly upregulated during metaphase I and RNAi mediated PRKAR2b depletion results in failure of MI progression [86].

The \textit{PATL2} gene has recently been demonstrated as another essential factor for MI progression [87]. Biallelic \textit{PATL2} mutations in women resulting in complete loss of the protein display germinal vesicle arrest, while oocytes of compound heterozygous patients with less severe mutations effectively make it through MI. However, fertilization rates are poor and the small number of embryos that are obtained, fail in early development [87]. Relatively little is currently known about the function of PATL2. RNAseq experiments in \textit{PATL2} \textit{-/-} murine oocytes have revealed a crucial role in the transcriptional regulation of oocyte maturation genes both in the germinal vesicle and during MII. One of the transcripts which is markedly downregulated in \textit{Patl2} mutated mouse oocytes is Cdc25a, which has also been shown to be crucial for meiotic progression [88, 89]. In line with this finding, translational regulation during oocyte maturation has been shown to be under

https://mc.manuscriptcentral.com/jmedgenet
control by the CPEB1 and DAZL proteins, which are responsible for ribosome loading onto oocyte specific mRNAs [90]. Additional transcriptional control in oocyte development has been observed for the FIGLA gene. Female Figla null mice display overexpression of testes specific genes in their ovaries [91]. Correspondingly, FIGLA mutations have been described in women with POI [92, 93].

In mice, transcriptional modulation of oocyte specific genes, including Dazl, Figla and Nobox, is under control by the master regulator Taf4b which associates with their respective proximal promoter sequence [94]. Consequently, mice deficient for Taf4b have oocytes displaying failure of prophase I progression going together with failure in synapsis. In conclusion, while the genes described in this section are necessary for meiotic progression, their molecular role is diverse, ranging from cell cycle control to transcriptionally initiating and fine-tuning oogenesis. This varied repertoire in functionality strongly suggests that still more genes are awaiting to be uncovered as essential for meiotic progression. A further possible implication could be that in certain patients, a multigenic origin can be consequential for their phenotype.

- Paracrine regulation of female meiosis

In mammalian oocytes, meiosis is arrested at the diplotene stage until the time of ovulation. Only by an increase of pre-ovulatory levels of luteinizing hormone (LH) can meiotic resumption proceed. LH acts on the outer granulosa cells and initiates a signaling cascade which has to reach the oocyte, which is separated from the outer surface of the follicle by more than 10 cell layers [95]. The LH signal transmission and subsequent control of meiotic progression is based on cGMP diffusion through these different layers. High levels of cGMP in the oocyte results in a meiotic arrest. However, the genes NPR2 and NPPC which are responsible for cGMP production are only expressed in the granulosa cells and thus diffusion is necessary in order to obtain high cGMP levels in the oocytes. In the oocyte, cGMP inhibits phosphodiesterase 3A (PDE3A) activity, suppressing cAMP hydrolysis leading to a subsequent activation of protein kinase A (PKA), which modulates the cell cycle [96, 97]. The dependence of the meiotic arrest on the presence of cGMP has been demonstrated in NPR2 null mice, which are infertile due to premature meiotic resumption [98]. The importance of diffusion has on the other hand been evidenced in connexin 37 deficient female mice, which are infertile due to an inhibition of meiotic completion [99].

Connexin proteins assemble into gap junctions which are widely expressed in different cell types. In follicular tissue, connexin 37 is responsible for diffusion at the oocyte-granulosa boundary, while connexin 43 based gap junctions form the connection between the granulosa cells. Interestingly, tissue specific overexpression of connexin 43 in connexin 37 deficient mice can restore oocyte maturation resulting in fertile female mice [100]. Currently, two modes of action have been described which contribute to the control of the meiotic arrest under the influence of LH. Murine follicles exposed to LH, show a significant decrease in estrogen receptor (ER) levels. Binding of
ER to the NRP2 and NPPC promotor subsequently leads to their expression. Therefore, reduced ER levels results in lower cGMP levels, as such permitting meiotic progression [101]. In a second study, it has been demonstrated that LH results in a significantly reduced permeability of the connexin 43 gap junctions in a MAPK dependent way [97]. As such, cGMP produced in the granulosa cells diffuses less efficiently to the oocyte, allowing the meiotic process to proceed.

While signaling from the granulosa cells towards the oocyte is crucial for follicular development, paracrine effects in the opposite directions play a key role as well. Oocyte expression of the TGFβ family member proteins GDF9 and BMP15 is essential for granulosa cell development [102, 103]. Binding of both proteins to the BMPRI and II receptor expressed on the granulosa and cumulus cells occurs from early folliculogenensis on and both proteins have been shown to interact with each other, forming the heterodimer cumulin, an activator of cumulus cells which is more potent than BMP15 or GDF9 alone [104]. GDF9 deficient mice are only able to form primary one-layer follicles [105]. Interestingly, GDF9 null oocytes grow faster and larger than controls despite incomplete follicle formation, but nevertheless show abnormalities including the absence of cortical granules and aberrant clustering of organelles around the germinal vesicle [103, 106]. Regulation of GDF9 expression is under control of the transcription factor NOBOX, and mutations in both GDF9 and NOBOX have been shown to lead to POI in humans [107–111]. Furthermore, NOBOX has been shown to interact with the FOXL2 transcription factor, in which mutations of the corresponding gene result in the blepharophimosis-ptosis-epicanthus inversus syndrome, which is associated with POI as well [112]. Additionally, mutations in BMP15 have been shown to lead to POI, suggesting that a disturbed BMP15-GDF9 interaction is contributive to the phenotype [108]. Furthermore, regulation of BMP15 expression has recently been found to be influenced by BNC1 (basonuclein 1) expression [113]. BNC1 mutations have been found in POI patients and resulted in reduced BMP15 expression in combination with meiotic defects in a mouse model.

**Maternal-effect factors**

The term ‘maternal-effect factor’ refers to maternally-encoded gene products, typically expressed in her oocytes, defects which do not affect her health but compromise the development of her offspring. The majority of maternal-effect genes have been studied using mouse models, but similar mutations are now being detected in humans, in rare, clinically-driven genomewide analyses. However, their prevalence and impact are not known in the wider landscape of clinical reproductive medicine [114, 115].

Some maternal-effect mutations directly affect the genome of the oocyte, and specifically the chromosome complement it delivers to the offspring. For example, a specific tubulin isoform, encoded by TUBB8, is required for the oocyte meiotic spindle, and maternal-effect mutations in TUBB8 can cause critical chromosomal defects affecting both oocytes and, remarkably, very early
development of fertilised embryos [116, 117].

The majority of maternal-effect mutations affect proteins or mRNA deposited in the oocyte which are required for post-fertilisation development. Upon fertilisation, the sperm genome enters the oocyte, and this triggers the completion of oocyte meiosis. The zygote then restructures and activates its genome through a co-ordinated sequence of functions, both epigenetically (changing the organisation of the zygote genome, and in particular the methylation of genomic DNA) and transcriptionally (potentiating expression of zygotic genes) (Fig. 2).

**Epigenetic:** The DNA methylation of oocyte and sperm are highly divergent, reflecting their highly differentiated state and gene expression patterns [118, 119], but these patterns are essentially harmonised by the time of blastulation (Fig. 2) [120, 121]. In the one-cell zygote, the paternal genome is rapidly and actively demethylated, [120–123], and appears to be predominantly passive, by reduction without replacement of DNA methylation over multiple cell cycles, possibly through restricted activity of the critical DNA methyltransferase DNMT1 [124, 125]. By the blastula stage, the DNA methylation of the two genomes is broadly equivalent, and low, with two exceptions. Firstly, constitutive heterochromatin and repetitive DNA are highly methylated and transcriptionally repressed after a brief zygotic window of transcription [126]. Secondly, a small number of sequences elude both DNA demethylation and remethylation, and thus retain the methylation state of their gamete of origin, in a phenomenon known as genomic imprinting [127].

**Transcriptional:** The maturing oocyte accumulates significant stocks of RNA and proteins; but the mature oocyte silences transcription [128], and remains transcriptionally dormant until full zygotic genome activation (ZGA) at 2C in mice, and the eight-cell stage (8C) in humans [129]. mRNA is very stable in the growing oocyte (with an average half-life of 10-14 days). On meiotic maturation, the average mRNA half-life returns to the normal level, of minutes or hours, and the mRNA content of the oocyte rapidly drops [130]. The progressive destabilisation of maternal mRNA, by removal of 5’ caps and shortening of 3’ polyA tails, is believed to contribute to oocyte ‘ageing’, depriving zygotes of maternal mRNA necessary for early development, and reducing their fitness [131–133].

- Maternal-effect genes and the zygote genome

During the remodelling of the embryonic genome, maternal-effect factors are essential, including not only epigenetic factors directly required for remodelling the genome, but also auxiliary factors that organise, stabilise and co-ordinate the use of maternally-provided RNA and protein until ZGA. Epigenetic factors in the oocyte are also universally required in somatic cells, and thus highly-penetrant mutations are incompatible with life; therefore maternal-effect mutations are not readily found in human populations, and their effects have been explored in mouse models. For example, Trim28 forms a scaffold, linking DNA-binding zinc-finger proteins with DNA demethylases and chromatin modifiers. Ablation of oocyte Trim28 expression caused complete lethality: the majority of embryos failed around blastulation, and fetuses surviving beyond this time showed gross
anatomical abnormalities. Interestingly, maternal null fetuses showed variably altered expression and DNA methylation of imprinted genes, suggesting that the lack of Trim28 in the first cell cycles exposed their differentially-methylated regions (DMRs) to demethylation, which was not restored during later development [134]. Remarkably, in both mice and humans TRIM28 haploinsufficiency (in either maternal or paternal inheritance) predisposes to perturbed imprinted gene expression, particularly in adipose tissue, and resultant obesity [135, 136]. It remains to be determined whether more severe hypomorphism for TRIM28 is associated with reproductive compromise.

DNMT1, which methylates hemimethylated DNA, has both somatic and oocyte-specific isoforms. In mice, absence of maternally-expressed Dnmt1 caused almost complete lethality of offspring, around mid-gestation, with a range of phenotypic abnormalities and DNA methylation defects, again including imprinted genes [124, 125, 137]. Maternal haploinsufficiency for Dnmt1 has also been shown to compromise offspring outcomes and DNA methylation, though only in presence of another environmental challenge, assisted reproductive technology [138]; but to date human mutations have not been reported.

Another critical epigenetic factor is the zinc-finger DNA binding protein ZFP57. In mouse models, Zfp57 binds to a hexamer motif in hemimethylated DNA, which recruits Trim28 and thereby Dnmt1 to facilitate maintenance DNA methylation [139, 140]. Combined maternal and zygotic knockout of Zfp57 in mouse results in loss of imprinted DNA methylation, and midgestation lethality [141]. Human ZFP57 is not a maternal-effect gene: it is expressed in the embryo, and somatic mutation carriers show imprinting disturbance and a congenital imprinting disorder, whereas maternal mutation carriers do not [142]. Maternally-provided Dppa3 (also known as Pgc7 or Stella) is essential for protection of methylation in the early murine embryo [143] but currently its human homologue is not associated with any reproductive phenotype.

- Maternal-effect genes and developmental competence

Imprinting disturbance is a recurring theme in the second class of maternal-effect mutations: those whose role may not be directly genomic, but possibly epigenomic or organisational. The archetype of these is mutation in NLRP7.

Human NLRP7 has no murine homologue. It was identified as a maternal-effect gene through mutations in mothers causing a severe adverse reproductive outcome, complete hydatidiform mole. However, heterozygous maternal mutations have been identified in the mothers of adverse reproductive outcomes, or offspring with altered DNA methylation [144–146]. Molar pregnancies do not produce liveborn offspring, but disorganised tissue resembling extraembryonic structures. The majority are sporadic, monospermic pregnancies with no maternal contribution; but women with homozygous NLRP7 inactivation, through mutation or gene deletion, show almost complete penetrance of molar pregnancy [147–149]. NLRP7-associated moles have a normal biparental
chromosome complement, but complete loss of DNA methylation on maternally-methylated imprints [150]. Molar pregnancies also result from maternal-effect mutations of *KHDC3L* [151], whose protein product associates with NLRP7 in the oocyte [152].

NLRP7 is one of a gene family, several of which are tandemly located and the products of recent duplication in mammalian evolution [153]. Several NLRPs are involved in humoral immunity [154], while others are expressed almost exclusively and abundantly in the oocyte. *Nlrp5* (also known as Mater) was one of the first maternal-effect genes identified [155]. Along with four other factors, Padi6, Khdc3 (also known as Filia), Moep and Tle6, Nlrp5 is among the most highly-expressed proteins in the oocyte [156, 157]. These proteins form a very high molecular weight complex, identified in some reports as the subcortical maternal complex (SCMC) [141] and others as cytoplasmic lattices (CPL) [158]. Maternal ablation of murine Nlrp5 causes arrest at the 2-cell (2C) stage [155]. In these maternal-null zygotes, CPL are not formed, and the majority of oocytes do not attain the ‘surrounded-nucleolus’ confirmation associated with early viability [158]. Khdc3l and Moep both have RNA-binding domains and RNA-binding activity in vitro [159]. Maternal-null *Khdc3l* mice have 50% fertility, with abnormalities of spindle assembly and chromosome alignment that cause delayed mitosis and gross aneuploidy [160]. Maternal null *Moep*/-/- embryos show delayed and asymmetric cell division resulting in arrest at 2C-4C. Padi6 interacts with the mitotic spindle and actin cytoskeleton of the oocyte, as well as with ribosomes; maternal ablation leads to disappearance of CPL, altered localisation of ribosomal components, reduced protein translation, reduced PolII transcription and developmental arrest at 2C-4C [161]. Tle6 is a phosphorylation target of PKA in oocyte maturation [162] but its function is not known. In humans, maternal-effect mutations have to date been identified in all these factors (Please add reference: Maternal variants in NLRP and other maternal effect proteins are associated with multilocus imprinting disturbance in offspring [163]. Inactivating mutations of *PADI6* and *TLE6* were found in mothers undergoing IVF for infertility, whose embryos arrested at 2C [164, 165]; *KHCD3L* mutations have been shown to cause familial hydatidiform mole. *NLRP5* variants caused a range of developmental outcomes, including infertility, molar pregnancy, miscarriage, and liveborn children affected by diverse imprinting disorders, and atypical imprinting disorders were also described in offspring of a mother with *NLRP2* mutations [166]. Other maternal-effect genes identified through murine studies but without currently-identified human effects include *Hsf1* [167], *Npm2* [168] and *Zfp36l2* [169]. Detailed characterisation of maternal-effect mutations in appropriate model systems is needed to reveal their mechanisms. It is plausible that complete or near-complete loss of function would cause zygote arrest before ZGA, and apparent infertility. It is furthermore very likely that environmental, medical, genetic and epigenetic problems all contribute to infertility and reproductive wastage; but their relative contributions are unclear.
**Phenotype selection**

Errors in MI could lead to an outcome of POI: the impossibility to proceed to MI might trigger an apoptotic effect in the immature oocytes, whereas failure to stop the meiotic cycle after completion of MI has been shown to lead to a premature depletion of the oocyte pool. Mutations in genes implicated in the formation of double-strand breaks, chromosome synapsis, homologous recombination and separation of homologous chromosomes, which are the main processes occurring during MI, could therefore potentially be involved in patients with a POI phenotype. Alterations in genes regulating maternal effect processes are expected to result in embryos which halt further development at a certain (early) stage. Moreover, and especially in an IVF setting, aberrations in maternal effects might lead to an increase in low quality embryos as well.

Additionally, errors in the mechanisms spanning the timeframe between ovulation and completion of MII post fertilization, could lead to a reduced fertilization rate or failure of the embryo to further develop. It has to be noted however that diminished fertilization can have other causes as well, ranging from paternal effects to defects in the acrosomal reaction, processes which are not included in this review.

In an ART/IVF clinical setting, defects in meiosis or maternal effect genes are expected to give rise to a specific phenotype. We therefore propose to initiate gene panel testing in patients with the following characteristics in the IVF clinic 1) oocyte maturation rate lower than 20% in the absence of endocrinological or technical issues in normal responders. 2) fertilisation rate lower than 10% in the absence of overt male factor. 3) embryo development rate lower than 10% in the absence of lab issues. However, prior setting these criteria, severe parental phenotypes (including immune problems) and high levels of sperm damage should be excluded. Sperm parameters including concentration, motility and morphology have been associated with the success in clinical pregnancies after ICSI [170]. We suggest to take into account the parameters proposed by the WHO as initial cut-off values [171]. Contrastingly, the presence of high sperm DNA damage has not been unambiguously shown as a significant success parameter during ICSI [172]. Furthermore, the couple should have been checked for karyotype errors. The presence of balanced translocations impact heavily on meioisis leading to chromosomal imbalances in the gametes. Likewise, Robertsonian translocations and aneuploidies for the sex chromosomes should be excluded as well. Ideally, mosaic chromosomal abnormalities should be excluded as well. In summary, when these parameters are considered, we estimate that the contribution of meiotic or maternal effect processes is likely.

**Concluding remarks**

The last decade has shown a significant increase in the genetic and molecular characterization of
fertility related processes and has given us a more clear insight in the cellular machinery which
drives meiosis and maternal effect processes. This research has predominantly been done in yeast
and mice and has revealed a myriad of novel proteins, both species specific and evolutionary
conserved, adding further to the complex regulation of these processes. Given the molecular
complexity of the meiotic process and its regulation, it is to be expected that multigenic alterations
or polymorphisms could lead to gradation of an infertility phenotype resulting from a deregulated
meiotic process. It is however unlikely that the use of a targeted gene panel will be able to identify
these subtle effects. In order to accomplish this, one would need a much more detailed description
of the phenotype as well as a large enough amount of samples with similar phenotype. However,
by using a meiotic gene specific panel in combination with a highly specific phenotype which is
readily identifiable by fertility centers, one can hope to further uncover the contribution of single
genes and as such identify the underlying cause of infertility of a proportion of idiopathic patients.
Furthermore, this would greatly improve our understanding of the meiotic / maternal effect process
and bring into view the impact certain genes have on the severity of the phenotype. More
importantly in terms of clinical practice, this would aid patients in their treatment regime as well as
patient families in terms of counseling.

Meiosis heavily depends on the formation and repair of double stranded breaks and mutations in
genes that are implicated in this process and have been associated with cases of familial cancer.
When mutations are found in any of these particular genes, the consulting physician or the fertility
center should have implemented well considered scenarios into their counseling practice. This is
however complicated by the fact that while for some repair genes, for instance BRCA1 and BRCA2,
the connection with familial breast and ovarian cancer is clear, while for other repair genes, this is
much less clear or even unknown at the moment. One approach to avoid this ethical issue is to
simply omit the repair genes in the panel. Whether the benefits of this approach outweigh the
disadvantages should be decided by the individual fertility centers in close collaboration with
ethicists.

In this review we have described candidate genes involved in two cellular processes, namely
meiosis and maternal effects, which are eligible for playing a role in specific cases of idiopathic
infertility. By using this set of genes in a diagnostic grade panel in combination with a specifically
selected phenotype, may improve the diagnosis for idiopathic infertility patients who fall into the
selected category. We realize that our gene set is not complete from a biological point of view.
However, in terms of clinical applicability the future implementation of a limited gene panel can
bring a significant benefit to the follow-up, treatment and counseling of patients. An overview of the
different genes described can be found in additional Table 1.
Figure 1: Overview of critical processes during the MI stage. A) After DNA replication, sister chromatids of both homologous chromosome pairs are held together by multiple units of the cohesin complex. B) Alignment of the homologous chromosomes is facilitated by the synaptonemal complex. C) The first step of homologous recombination occurs through the formation of double strand breaks (DBS). This process is Spo-11 dependent and strand invasion is mediated by the Rad51-DMC1 complex which is stabilized by Hop2-Mnd1. D) After homologous recombination, the cohesin complex of the sister chromatids is cleaved by separase along the length of the sister chromatids. Cohesin at the centromeres is protected by shugosin, inhibiting the separase mediated cleaving. E) Sister kinetochores connect to microtubules emanating from the same spindle poles, as such separating the newly recombined homologues.
**Figure 2**: Overview of the general methylation and transcriptional status of the oocyte, zygote and further developmental stages. From fertilization on, the paternal DNA is actively demethylated. Demethylation of the maternal DNA occurs more passively, being not replaced during initial cell divisions. From the blastocyst stage on, expression of DNMT1 increases, which goes together with an increase of methylation of the embryonic DNA. Transcripts originating from the oocyte are very stable and constitute most of the mRNA during initial stages. However, from the 4-8 cell stage on, embryonic transcripts take over.
References


31 Tenenbaum-Rakover Y, Weinberg-Shukron A, Renbaum P, Lobel O, Eideh H, Gulsuner S,


46 Bolcun-Filas E, Rinaldi V, White M, Schimenti J. Reversal of female infertility by Chk2


51 Bugreev DV, Huang F, Mazina OM, Pezza RJ, Voloshin ON, Daniel Camerini-Otero R, Mazin AV. HOP2-MND1 modulates RAD51 binding to nucleotides and DNA. Nat Commun 2014;5:4198.


https://mc.manuscriptcentral.com/jmedgenet


82 Oh JS, Susor A, Conti M. Protein tyrosine kinase Wee1B is essential for metaphase II exit in mouse oocytes. *Science* 2011;332:462–5.


152 Akoury E, Zhang L, Ao A, Slim R. NLRP7 and KHDC3L, the two maternal-effect proteins responsible for recurrent hydatidiform moles, co-localize to the oocyte cytoskeleton. *Hum Reprod* 2015;30:159–69.


162 Duncan FE, Padilla-Banks E, Bernhardt ML, Ord TS, Jefferson WN, Moss SB, Williams CJ. Transducin-like enhancer of split-6 (TLE6) is a substrate of protein kinase A activity during mouse oocyte maturation. *Biol Reprod* 2014;90:63.


170 Zhang J, Xue H, Qiu F, Zhong J, Su J. Testicular spermatozoon is superior to ejaculated spermatozoon for intracytoplasmic sperm injection to achieve pregnancy in infertile males with high sperm DNA damage. *Andrologia* 2018;e13175.


Overview of critical processes during the MI stage. A) After DNA replication, sister chromatids of both homologous chromosome pairs are held together by multiple units of the cohesin complex. B) Alignment of the homologous chromosomes is facilitated by the synaptonemal complex. C) The first step of homologous recombination occurs through the formation of double strand breaks (DSB). This process is Spo-11 dependent and strand invasion is mediated by the Rad51-DMC1 complex which is stabilized by Hop2-Mnd1. D) After homologous recombination, the cohesin complex of the sister chromatids is cleaved by separase along the length of the sister chromatids. Cohesin at the centromeres is protected by shugosin, inhibiting the separase mediated cleaving. E) Sister kinetochores connect to microtubules emanating from the same spindle poles, as such separating the newly recombined homologues.
Overview of the general methylation and transcriptional status of the oocyte, zygote and further developmental stages. From fertilization on, the paternal DNA is actively demethylated. Demethylation of the maternal DNA occurs more passively, being not replaced during initial cell divisions. From the blastocyst stage on, expression of DNMT1 increases, which goes together with an increase of methylation of the embryonic DNA. Transcripts originating from the oocyte are very stable and constitute most of the mRNA during initial stages. However, from the 4-8 cell stage on, embryonic transcripts take over.
<table>
<thead>
<tr>
<th>Meiosis genes</th>
<th>Maternal effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>DNMT1</td>
</tr>
<tr>
<td>ATR</td>
<td>DPPA3</td>
</tr>
<tr>
<td>BMP15</td>
<td>HSF1</td>
</tr>
<tr>
<td>BMPRI</td>
<td>KHDC3L</td>
</tr>
<tr>
<td>BMPRII</td>
<td>KHDC3L</td>
</tr>
<tr>
<td>BNC1</td>
<td>MOEP</td>
</tr>
<tr>
<td>BRCA1</td>
<td>NLRP2</td>
</tr>
<tr>
<td>BRCA2</td>
<td>NLRP5</td>
</tr>
<tr>
<td>CDK1</td>
<td>NLRP7</td>
</tr>
<tr>
<td>CDK2</td>
<td>NPM2</td>
</tr>
<tr>
<td>CHEK1</td>
<td>PADI6</td>
</tr>
<tr>
<td>CHK2</td>
<td>TLE6</td>
</tr>
<tr>
<td>CKS1</td>
<td>TRIM28</td>
</tr>
<tr>
<td>CKS2</td>
<td>TUBB8</td>
</tr>
<tr>
<td>GJA4</td>
<td>ZFP36L2</td>
</tr>
<tr>
<td>GJA1</td>
<td>ZFP57</td>
</tr>
<tr>
<td>CPEB1</td>
<td></td>
</tr>
<tr>
<td>DAZL</td>
<td></td>
</tr>
<tr>
<td>DMC1</td>
<td></td>
</tr>
<tr>
<td>EMI2</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>FIGLA</td>
<td></td>
</tr>
<tr>
<td>FOXL2</td>
<td></td>
</tr>
<tr>
<td>GDF9</td>
<td></td>
</tr>
<tr>
<td>H2AFX</td>
<td></td>
</tr>
<tr>
<td>HFM1</td>
<td></td>
</tr>
<tr>
<td>HOP2</td>
<td></td>
</tr>
<tr>
<td>HORMAD1</td>
<td></td>
</tr>
<tr>
<td>HORMAD2</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td></td>
</tr>
<tr>
<td>LHX8</td>
<td></td>
</tr>
<tr>
<td>MCM8</td>
<td></td>
</tr>
<tr>
<td>MCM9</td>
<td></td>
</tr>
<tr>
<td>MEIKIN</td>
<td></td>
</tr>
<tr>
<td>MEIOB</td>
<td></td>
</tr>
<tr>
<td>MND</td>
<td></td>
</tr>
<tr>
<td>MRE11</td>
<td></td>
</tr>
<tr>
<td>MSH4</td>
<td></td>
</tr>
<tr>
<td>MSH5</td>
<td></td>
</tr>
<tr>
<td>NBS1</td>
<td></td>
</tr>
<tr>
<td>NOBOX</td>
<td></td>
</tr>
<tr>
<td>NPPC</td>
<td></td>
</tr>
<tr>
<td>NPR2</td>
<td></td>
</tr>
<tr>
<td>PALB2</td>
<td></td>
</tr>
<tr>
<td>PATL2</td>
<td></td>
</tr>
<tr>
<td>PDE3A</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td></td>
</tr>
<tr>
<td>PKA</td>
<td></td>
</tr>
<tr>
<td>PP2A</td>
<td></td>
</tr>
</tbody>
</table>
PRDM9
RAD50
RAD51
REC11
REC8
SCC3
ESP1
SET
SGO2
SGOL1
SMC1
SMC1b
SMC1b
SMC3
SOHLH2
SPO11
STAG1
STAG2
STAG3
SYC2PL
SYCE1
SYCP2
SYCP3
TP53
WEE2
SGOL2
TAF4B
CDC25a
PRKAR2B
ME1
SPATA22
MEIOB
MOS