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Primary ovarian insufficiency, meiosis and DNA repair

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ABSTRACT

Premature ovarian insufficiency (POI) is a major cause of female infertility. It is a heterogeneous disease that affects about 1% of women under 40 years of age. POI may be due to abnormal follicle stock formation, increased follicular atresia, impaired recruitment of dominant follicles, blocked follicular maturation or rapid depletion of the follicular stock. It remains idiopathic in most cases but the existence of familial cases shows that it can have a genetic origin. Next generation sequencing (NGS) strategies have allowed the identification of new genes involved in the etiology of POI. Here, I briefly describe some studies demonstrating that pathogenic variants in ‘DNA repair and meiotic genes’ underlie POI. Some of the examples show the power of the combination of classical genetics and NGS in the discovery of novel ‘POI genes’.

The genetics of primary ovarian insufficiency: back to basics

Premature ovarian insufficiency (POI) is a major cause of female infertility. This condition is characterized by an early arrest of menstruation and high follicle stimulating hormone (FSH) levels before the age of 40 years. It can occur during the peri-pubertal period along with primary amenorrhea or appear later (secondary amenorrhea) [1]. It is a highly

heterogeneous disorder that affects about 1% of women under 40 [2]. Several mechanisms have been proposed to explain the ovarian defects underlying POI: abnormal formation of the follicle stock or its rapid depletion, increased follicular atresia, impaired dominant follicle recruitment, block of follicular maturation, etc. Patients with POI have an often definitive infertility associated with decreased levels of inhibin B, anti-Müllerian hormone and estrogens, as well as, increased gonadotropins levels [3,4]. Different etiologies of POI have been

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identified: it can be secondary to chemotherapy or radiotherapy, viral, autoimmune or genetic.

The fact that up to 31% of subjects with POI have at least one affected relative highlights the existence of a genetic component [5]. Thus far, cytogenetics, cytogenomics (array CGH) and high throughput sequencing approaches have uncovered genetic causes in about 20–25% of POI cases [6]. A number of pathogenic variants in genes encoding signaling molecules, transcription factors and gonadotropin receptors have been identified [7]. POI may be syndromic, as in the case of Turner or the blepharophimosis syndromes [4,8–10]. Despite the progresses made in the field, the known genetic causes explain only a small percentage of cases of POI [7] pointing to a large number of underlying genes/loci (i.e., genetic heterogeneity) and/or to complex modes of inheritance (i.e., digenic or oligogenic) [11].

Recent years have witnessed the advent of next generation sequencing (NGS), which has allowed the identification of a plethora of genes involved in the etiology of many genetic conditions including POI. The existence of familial cases of POI has been instrumental in this process because NGS coupled to classical linkage analysis or homozygosity mapping can lead to the unequivocal identification of causal pathogenic variants. Linkage analysis is based on the study of the co-segregation of alleles of genetic markers scattered throughout the genome with the disease. This allows to pinpoint the regions of the genome that may contain the pathogenic variant [12]. In turn, homozygosity mapping is based on the detection of the disease locus within a region expected to be homozygous by descent in affected patients within consanguineous families [13].

Consanguineous families are particularly interesting to uncover POI-causing variants using NGS, be it by whole exome sequencing (WES) or whole genome sequencing (WGS). WES and WGS make it possible to gain access either to the sequence of the coding part of the genome or to the whole, respectively. For relatively large consanguineous families, variant filtering is utterly simple. Indeed, the candidate causal

variants should be present in a homozygous state in the patients and be either heterozygous or absent in unaffected relatives (the parents being heterozygotes, Fig. 1). In more general terms, in cases of recessive POI, affected women are expected to be compound heterozygotes for the disease-causing variants.

Here, I describe a series of selected examples showing the involvement in POI of pathogenic variants affecting genes encoding DNA repair and meiotic factors.

Meiotic chromosome pairing, synapsis and POI

At the onset of meiosis the homologous chromosomes have to become physically close before they can exchange DNA by recombination between the paternal and maternal chromosomes. The synaptonemal complex (SC) is a zipper-like protein structure that appears between homologous chromosomes during meiosis. Specifically, it maintains paired homologous chromatids ensuring interchromatid exchanges. Three main components of the SC have been identified: Synaptonemal Complex Protein-1 (SYCP1), SYCP2, and SYCP3 [14,15].

Cohesins form a ring-shaped protein structure essential for chromosome pairing during meiosis. The cohesin ring surrounds the chromatids contributing to the establishment of the SC. Therefore, the absence of a component of the ring may lead to a mismatch of the homologs, meiotic arrest and degeneration of the oocytes and eventually of the ovary [16]. The analysis of a consanguineous family in which a recessive form of POI segregates allowed us to implicate a variant affecting a component of the cohesin ring as responsible for the disorder. In brief, classical linkage analysis pointed to three candidate regions supposed to contain the causal variant [17]. Then, WES pointed to a frameshift variant in Stromal Antigen 3 (STAG3). Both copies were affected in the patients with POI and, as expected, the parents were heterozygous [Fig. 2]. The fertile siblings were either heterozygous or homozygous for the normal allele [18]. Many subsequent studies have confirmed the causal role of STAG3 mutations in POI [19–23]. The knock-out of *Stag3* in mouse leads to both female and male sterility. Oocytes are blocked in early meiosis, which leads to their massive degeneration during the first week after birth [18]. Further studies have confirmed such results [24–26]. Male mice lacking *Stag3* are azoospermic [27] and this is also the case in human males [28]. The involvement of STAG3 in infertility extends the list of cohesinopathies. Such pathologies, as the Cornelia de Lange or Roberts syndromes, are characterized by defects in fetal and post-natal development, congenital malformations, growth retardation and intellectual disability [29]. Isolated infertility in the case of the STAG3 pathogenic variants is explained by its ‘purely’ meiotic expression.

Alterations of the SC itself can also be responsible for POI. Indeed, a genetic study based on NGS has described a homozygous truncating variant of Synaptonemal Complex Central Element Protein 1 (SYCE1) in sisters with POI in a

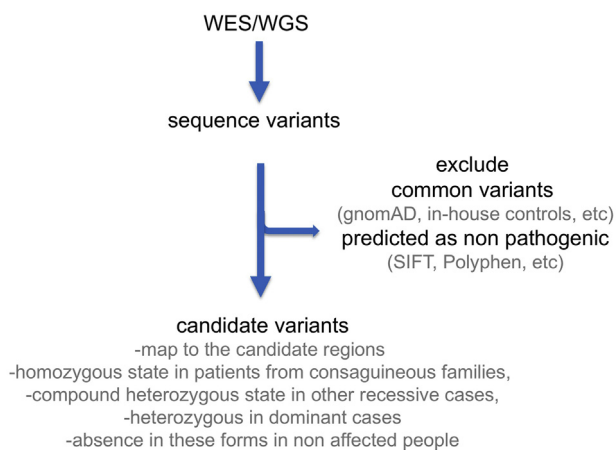


Fig. 1 Outline of variant filtering when using Next Generation Sequencing. gnomAD (<https://gnomad.broadinstitute.org>).

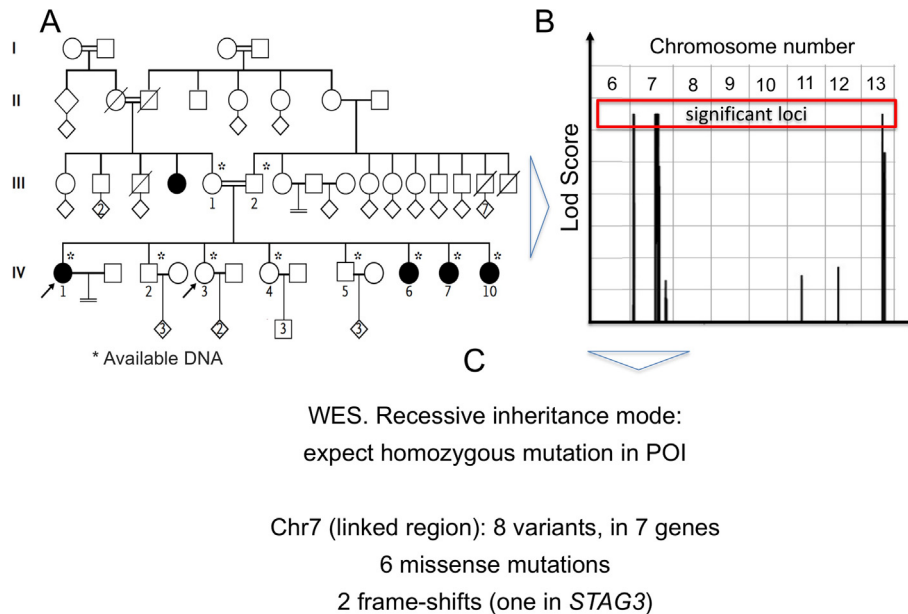


Fig. 2 Example of the implication of a *STAG3* variant in POI.

consanguineous family. The parents and tested brothers were heterozygous for the variant and it was absent from the unaffected sister tested [30]. Such results are consistent with the infertility observed in animal models deleted for genes involved in SC formation [31]. As expected, variants in *SYCE1* are also responsible for male infertility. Indeed, a study reported a homozygous *SYCE1* splice site variant resulting in intron retention and a subsequent frameshift in non-obstructive azoospermia segregating in a consanguineous Iranian Jewish family [32]. Thus far, *SYCE1* variants associated with infertility are consistent with a recessive inheritance mode [33]. In turn, several heterozygous *SYCP3* variants have been described and might exert a dominant-negative effect [33].

Alterations of other proteins of DNA recombination and repair

Homologous recombination (HR), which is essential for meiosis, is initiated by DNA double-strand breaks (DSB). The DSB sites are then recognized and partially degraded [Fig. 3]. This provides the substrate ensuring the search for homologous sequences between the synapsed chromatids. A post-synaptic phase requiring DNA synthesis also involves the resolution of the DNA exchange intermediates [14].

Minichromosome maintenance 8 (*MCM8*) and *MCM9* are members of the Mini Chromosome Maintenance family of proteins (*MCM2-9*) involved in HR. They share the conserved helicase domain of the *MCM* family able to open double-stranded nucleic acids. The *MCM2-7* complex, being the main replicative helicase, has been well characterized [34]. In contrast, the function(s) of *MCM8* and *MCM9* are less well known [35]. Female mice lacking *Mcm8* are sterile and their

ovaries are devoid of oocytes. In males, spermatocytes are blocked in meiotic prophase I [35]. In line with such observations, the analysis of sisters with primary amenorrhea from a consanguineous marriage having also hypothyroidism and hypergonadotropic hypogonadism revealed the presence of a missense variant (p.P149R) in *MCM8* [36]. This variant was found in a region of homozygosity present in the patients and not in unaffected sisters. Consistent with the involvement of the *MCM8* helicase in HR and in the repair of DSB, the fibroblasts of the sisters with POI were hypersensitive to chromosomal breaks compared to fibroblasts of unaffected sisters. Moreover, the recruitment of *MCM8* at sites of DNA breaks and its activity were found to be disrupted. This study identified the first pathogenic variant in *MCM8* being involved in

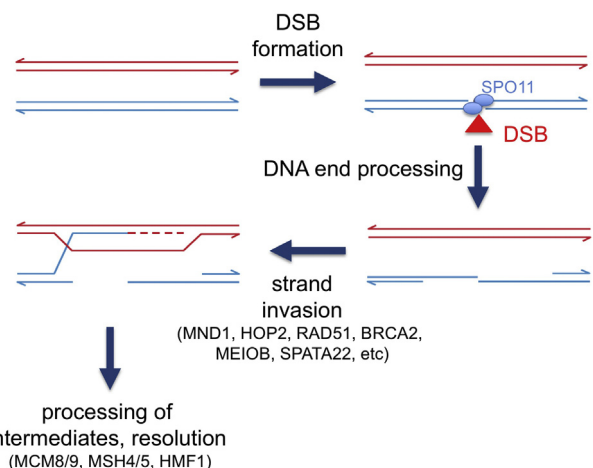


Fig. 3 Outline of the process of homologous recombination taking place during meiosis. The names of several gene products discussed in the text are mentioned but the list is not exhaustive.

autosomal recessive POI and has been corroborated by subsequent reports [37,38]. For instance, WES identified homozygous (splice and frameshift) variants in *MCM8* in two consanguineous families with cases of both male and female infertility [39]. As expected, mitomycin C (MMC) treatment elicited higher chromosomal breakage levels in cells in homozygous individuals than in controls [39].

Similarly, the absence of *Mcm9* in mice induces meiotic recombination defects and oocyte degeneration. However, the testes are able to produce a reduced amount of sperm that is still compatible with some degree of fertility [35]. As outlined below, several studies have found pathogenic variants of *MCM9* responsible for POI. For instance, the study of a family with POI patients affected with primary amenorrhea and small height identified a homozygous splice donor site variant, resulting in the production of truncated forms of *MCM9* that cannot be recruited at the sites of DNA damage [40]. In another family, a truncating variant (p.Arg132*) was supposed to result also in the loss of *MCM9* activity [40]. As in the case of the *MCM8* pathogenic variants, the repair of chromosomal breaks in patients' lymphocytes was impaired. The authors of this paper proposed that recessive pathogenic variants of *MCM9* would cause a syndrome associating genomic instability with hypergonadotropic hypogonadism and small height [40]. Using linkage analysis and homozygosity mapping of another consanguineous family, a subsequent study found a homozygous causal variant in *MCM9* (p.E495* at the protein level). This variant is also supposed to lead to a loss of function. However, in this family the affected sisters had a normal height. These results confirmed the implication of *MCM9* in POI and broadened the phenotypic spectrum to include patients with normal height [41]. This spectrum was further expanded with data demonstrating the presence of a homozygous pathogenic variant of *MCM9* in cancer-prone cases of POI [42].

In line with the results discussed above, disease-causing variants in the meiotic Helicase For Meiosis 1 (*HFM1*) gene, encoding another helicase necessary for HR, have been reported [43]. Specifically, WES of two sisters with POI and their parents led to the identification of pathogenic variants in a compound heterozygous state. A similar case was found in a cohort of patients with sporadic POI. These results suggest that biallelic variants of *HFM1* can be responsible for recessive POI [43]. Genetic associations between *HFM1* variants and both POI [44] and idiopathic azoospermia or severe oligozoospermia have also been reported [45].

More recently, WES of members of a Colombian POI family allowed the identification of a homozygous pathogenic variant affecting a donor splice site and thus altering splicing of the meiotic gene MutS homolog 4 (*MSH4*). Minigene assays showed that the mutation induced skipping of exon 7, which translates into p.Ile743_Lys785del at the protein level. The variant is expected to disrupt the ATP binding domain and it is therefore predicted to inactivate *MSH4*. Interestingly, the affected sisters presented with secondary amenorrhea, which suggests that either some residual *MSH4* activity is present or that, unlike in mouse [46], some degree of functional compensation by another *MSH* gene can take place. In line with the first possibility, the pathogenic variant transforms a splice site processed by the major spliceosome into one that

could be processed, albeit with much lower efficiency, by the minor (U12) spliceosome [47]. Thus, a small amount of normal mRNA and *MSH4* protein could in principle be produced *in vivo*. A mouse model of the human mutation would provide insights into its impact on meiosis and fertility. A pre-NGS analysis of a cohort of POI patients had identified a heterozygous variant in *MSH5* leading to the p.P29S substitution at the amino acid level [48]. However, this substitution is too frequent to be responsible (at least alone and in a heterozygous state) for POI. Indeed, according to gnomAD (<https://gnomad.broadinstitute.org>) it reaches 12% in the general population. More recently, a homozygous missense mutation (p.D487Y at the protein level) has been identified in *MSH5* in two sisters with POI. *In vitro* functional experiments have shown that mutated *MSH5* is impaired for repair by HR. Moreover, the knock-in of the mutation in mice leads to the presence of atrophic ovaries without oocytes. Several heterozygous mutations were identified in the same study but the current level of evidence is not enough to formally implicate them in POI [49].

Along a similar vein, WES of two Finnish sisters with non-syndromic POI allowed the identification of a homozygous truncating variant in Fanconi Anemia Complementation Group M (*FANCM*) leading to p.Gln1701*. *FANCM* is a DNA-damage response gene whose heterozygous variants predispose to breast cancer [50–52]. Accordingly, patients' lymphocytes displayed higher levels of basal and MMC-induced chromosomal aberrations compared to the mother's cells. The lymphoblasts of the former were also hypersensitive to MMC and MMC-induced monoubiquitination of *FANCD2* was found to be impaired. Expression studies showed that *FANCM* is preferentially expressed along the chromosomes in pachytene cells, those undergoing meiotic recombination. This pathogenic variant may provoke meiotic defects leading to a depleted follicular stock, as has been observed in *Fancm*^{-/-} mice [53]. Subsequent studies have implicated *FANCM* variants in mendelian male infertility [54,55]. Last year, compound heterozygous truncating mutations of Breast Cancer Type 2 (*BRCA2*, also known as *FANCD1*), leading to a reduction *BRCA2* protein levels and an impaired response to DNA damage were reported in two sisters with 46,XX ovarian dysgenesis. As expected, such mutations resulted in chromosomal breakage and in a failure of the recombinase *RAD51* to be recruited to DSBs [56]. In this report, infertility was accompanied by microcephaly and one of the patients was in remission from leukemia. A *Drosophila* model devoid of the *BRCA2* orthologue displayed gonadal dysgenesis in both sexes. Such results point to the importance of *BRCA2* in ovarian development and function [56]. Very recently, two heterozygous frameshift mutations, c.1048_1051delGTCT (p.Gln350Valfs*18) and c.739dupA (p.Met247Asnfs*4), were identified in *FANCL* gene in POI cases [57]. Mutated *FANCL* proteins were retained in the cytoplasm whereas the wild-type was predominantly nuclear. Moreover, the former exhibit impaired ubiquitin-ligase activity and compromised DNA repair ability after MMC treatment [57].

Meiosis specific with OB domain (*MEIOB*) encodes another factor essential for meiotic recombination, conserved among metazoans, which contains single-stranded DNA (ssDNA) binding domains similar to those of Replication protein A1

(RPA1). RPA1 is the large subunit of the ubiquitous single-stranded DNA-binding (SSB) protein complex RPA [58]. MEIOB binds to ssDNA during meiotic DSB repair and is required for proper meiotic recombination in complex with spermatogenesis associated 22 (SPATA22). Last year, we found a homozygous synonymous variant of MEIOB leading to impaired splicing, by WES of two sisters affected with POI within a consanguineous family [59]. The homozygous variant affected the last base of exon 12 and was predicted to strongly perturb pre-mRNA splicing. We assessed the impact of the identified MEIOB variant using a minigene assay and by sequencing illegitimate transcripts from the proband's leukocytes. Such experiments showed that the variant induced skipping of exon 12, which leads to the production of a C-terminally truncated protein unable to interact with its partner SPATA22. This abolishes their recruitment to DSBs. Such results are consistent with the depleted follicular stock observed in *MeioB*^{-/-} mice [58,60]. This is the first molecular defect reported in a meiosis-specific SSB protein responsible for POI. We hypothesize that alterations in other SSB proteins could explain other cases of syndromic or isolated ovarian insufficiency. Again, the affected sisters presented with a secondary amenorrhea suggesting that either there is some degree of leakiness (i.e., some normal splicing) or that this gene is not as essential for fertility as it is in mice. This calls for a mouse model to further investigate this variant. Several MEIOB variants, including a recurrent one, have been reported in male infertility [61,62].

It is also worth noting that a study using WES in a non-consanguineous family uncovered a heterozygous pathogenic variant in the PiggyBac Transposable Element Derived 3 (CSB-PGBD3) fusion gene [63]. This gene encodes a protein involved in DNA damage repair and Cockayne syndrome, a disease characterized by progressive neurological disorders, intellectual disability, facial dysmorphism, photosensitivity, etc. [64]. Classical sequencing of more than 400 sporadic cases led to the identification of two other pathogenic variants. Functional analyses showed that the mutated protein displayed an impaired recruitment to damaged sites, confirming the importance of DNA repair for normal ovarian function.

Before closing this section, it is also worth mentioning a study of a consanguineous family using homozygosity mapping and NGS that revealed a homozygous three-base pair deletion (NM_016556.2, c.600_602del) in PSMC3 Interacting Protein (PSMC3IP). This alteration leads to the deletion of a glutamic acid residue in the C-terminal domain of the protein [65]. PSMC3IP is the homologue of the HOMologous Pairing 2 (*Hop2*) protein in yeast. *Hop2* and its partner Meiotic Nuclear Divisions 1 (*Mnd1*) form a heterodimer necessary for homologous chromosome pairing and recombination. Indeed, the *Hop2*-*Mnd1* complex is responsible for the stimulation of the DNA Meiotic Recombinase 1 (*Dmc1*) and Rad51 recombinases [66]. Female mice devoid of *Psmc3ip/Hop2* display a significant reduction in ovarian size. Specifically, the female *Psmc3ip/Hop2* knockout shows ovarian tubulostromal hyperplasia with an absence of follicles. On the male side, profound meiotic defects are observed. Indeed,

Hop2^{-/-} male mice display a spermatocyte arrest at a pachytene-like stage. Axial elements are present but synapsis is limited. This leads to testicular hypoplasia and spermatogenesis blockade in meiosis I. In sum, the lack of mature gametes in both sexes of the *Psmc3ip/Hop2* knockout mice is consistent with a role for PSMC3IP/HOP2 in meiosis [67]. However, the possibility of a meiotic defect in the patients described above could not be directly assessed [65]. Thus, current data do not allow the authors to show a direct impact of the described pathogenic variant on the meiotic role of PSMC3IP. A subsequent study screened a group of 50 Swedish patients with POI for mutations in PSMC3IP. No alteration could be detected in this cohort showing that PSMC3IP pathogenic variants are a rare cause of POI [68]. A more recent study identified a truncating variant in a consanguineous POI family. A concomitant variant in the Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit (CLPP) gene was detected but it seems to be unrelated with POI in this specific family [69]. Interestingly, recent functional studies have demonstrated that a C-ter deleted form of PSMC3IP or even the variant p.Glu201del abolishes or decreases, respectively, the interaction with DMC1 and RAD51 [70]. A male homozygote in this consanguineous family had azoospermia, showing that infertility due to pathogenic variants in PSMC3IP can affect both sexes [69].

The same pre-NGS analysis of a cohort of patients with POI mentioned above had identified a homozygous

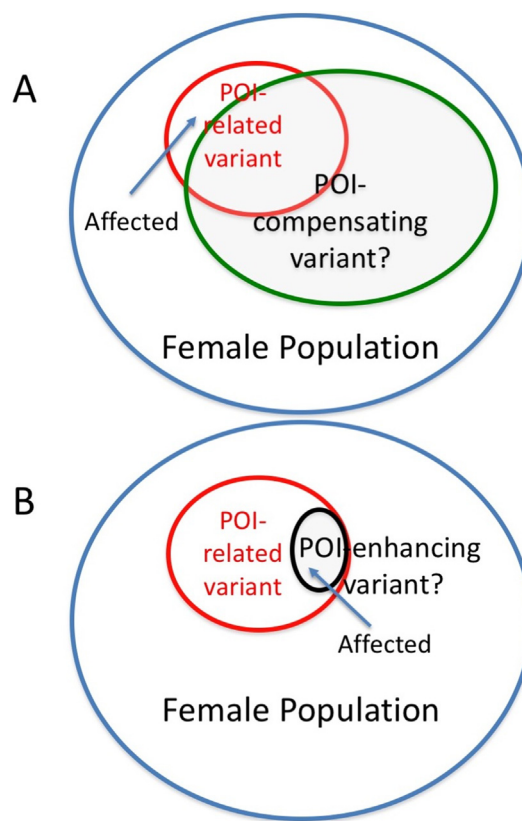


Fig. 4 Interloci interactions and POI.

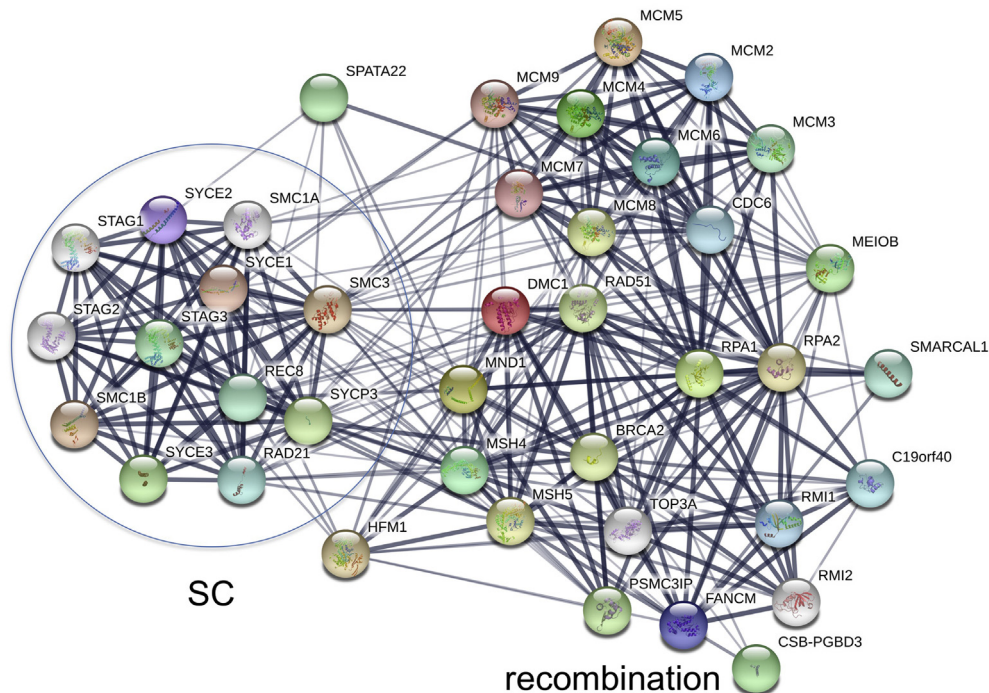


Fig. 5 Network of interaction of some proteins involved in meiosis and DNA repair. It involves those mentioned in the text or retrieved from the CORUM Database and further analysis using STRING [73]. The CORUM database, accessible at <http://mips.helmholtz-muenchen.de/genre/proj/corum/about/corum.html>, was queried with the word 'meiosis'. The following list (REC8, SMC1b, SMC3, SYCP3, STAG3, MND1, PSMC3IP, SYCE1, SYCE2, SYCE3, CSB-PGBD3, MCM8, MCM9, MCM2-7, SPATA22, MEIOB, FANCM, MSH4, MSH5, HFM1, SYCP3, DMC1, RAD51, BRCA2, RPA1-2) was submitted to STRING to generate a graphical representation. The analysis was based on textmining, experiments and databases (interaction score 0.4) and included up to 5 interactors of a given node not included in the initial list. The level of interaction evidence is given by the thickness of the edges.

nucleotide substitution in DMC1 leading to the M200V substitution at the protein level. The variant was found in a woman of African origin [48], a geographical region where this variant reaches a frequency of 12% according to gnomAD, an information which was not available at the time of the publication. According to our current knowledge, this and the fact that the variant is predicted to be tolerated by several bioinformatic tools suggest it is a poor candidate to explain POI. Despite these facts, the introduction of the corresponding variant in the *Schizosaccharomyces pombe* *dmc1* ortholog causes a significant decrease in meiotic recombination. A deleterious effect was also noticed in biochemical assays [71]. However, much more recently, a mouse knocked-in model of Dmc1-M200V has been generated. This study revealed that both female and male mice were fully fertile and did not display any gonadal abnormalities. This emphasizes the importance of animal models in the validation of gene variants involved in human infertility, even if such models can be sensitive to the genetic background [72]. This example points to the importance of the variant filtering step of any WES/WGS, which should take into account the frequency of the variant in the appropriate ethnic background to be meaningful. Indeed, a POI-related variant can be rare or even absent in several populations becoming a potentially good candidate on this ground. However, it can be frequent in another population

becoming a very poor candidate. This situation also allows us to speculate on the possibility of the existence of intergenic interactions. In fact, even for the variant underlying Dmc1-M200V, one cannot rule out, at least on formal grounds, the existence of a frequent variant (Fig. 4) present in the population able to compensate for the negative effects of the POI-related variant in homozygotes. Only women without the compensating variant would be affected by POI. Another possibility is the co-segregation of a POI-enhancing variant in affected women. Again, these examples are provided here for the sake of the argument, yet we have to keep in mind that this formal possibility exists.

Conclusions

The overview of recent and some less recent work provided above highlights several actors whose pathogenic variants are responsible for POI and reveal the importance of a proper level of genomic stability in establishing and maintaining fertility. They also show the power of formal genetics of consanguineous families allied to NGS in the discovery of 'new genes' involved in POI. Meiosis is a very complex process that involves dozens of candidate factors [14] whose pathogenic variants can explain cases of POI. The cases discussed above

are mostly Mendelian. However, it is likely that genome-wide analyses will uncover pathogenic variants in more than one gene, pointing to digenicity and perhaps oligogenicity. This might also contribute to explain the incomplete penetrance sometimes observed in carriers of well-known mutations involved in POI [11]. This is a very plausible possibility in the light of the complexity of meiosis and HR [Fig. 5]. Fig. 5 shows the high degree of connectivity of the protein–protein interaction network of factors involved in meiosis and HR. This may underlie genetic interactions of (mainly) heterozygous variants whose co-occurrence might be responsible for POI. Di/oligogenic inheritance is difficult to demonstrate on formal grounds and animal models will be helpful in the future because in some instances only an animal model can formally prove or disprove the causality of the relevant variants.

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Conflicts of Interest

The author has no conflict of interest to declare.

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