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Potential genetic causes of miscarriage in euploid pregnancies: A systematic review

Running title: Genetic causes of miscarriage in euploid pregnancies

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Abstract

BACKGROUND: Approximately 50% of pregnancy losses are caused by chromosomal abnormalities, such as aneuploidy. The remainder have an apparent euploid karyotype, but it is plausible that there are cases of pregnancy loss with other genetic aberrations that are not currently routinely detected. Studies investigating the use of exome sequencing and chromosomal microarrays in structurally abnormal pregnancies and developmental disorders have demonstrated their clinical application and/ or potential utility in these groups of patients. Similarly, there have been several studies that have sought to identify genes that are potentially causative of, or associated with, spontaneous pregnancy loss, but the evidence has not yet been synthesized.

OBJECTIVE AND RATIONALE: The objective was to identify studies which have recorded monogenic genetic contributions to pregnancy loss in euploid pregnancies, establish evidence for genetic causes of pregnancy loss, identify the limitations of current evidence and make recommendations for future studies. This evidence is important in considering additional research into Mendelian causes of pregnancy loss and appropriate genetic investigations for couples experiencing recurrent pregnancy loss.

SEARCH METHODS: A systematic review was conducted in MEDLINE (1946 to May 2018) and Embase (1974 to May 2018). The search terms “spontaneous abortion”, “miscarriage”, “pregnancy loss” or “lethal” were used to identify pregnancy loss terms. These were combined with search terms to identify the genetic contribution including “exome”, “human genome”, “sequencing analysis”, “sequencing”, “copy number variation”, “single nucleotide polymorphism”, “microarray analysis” and “comparative genomic hybridization”. Studies

were limited to pregnancy loss up to 20 weeks in humans, and excluded if the genetic content included genes which are not lethal *in utero*, PGD studies, infertility studies, expression studies, aneuploidy with no recurrence risk, methodologies where there is no clinical relevance and complex genetic studies. The quality of the studies was assessed using a modified version of the Newcastle-Ottawa scale.

OUTCOMES: A total of 50 studies were identified and categorized into three themes; whole exome sequencing studies, copy number variation studies and other studies related to pregnancy loss including recurrent molar pregnancies, epigenetics and mitochondrial DNA aberrations. Putatively causative variants were found in a range of genes, including cholinergic receptor, nicotinic, alpha polypeptide 1 (*CHRNA1*), dynein, cytoplasmic 2, heavy chain 1 (*DYNC2H1*) and ryanodine receptor 1 (*RYR1*), which were identified in multiple studies. Copy number variants were also identified to have a causal or associated link with recurrent miscarriage.

WIDER IMPLICATIONS: Identification of genes that are causative of or predisposing to pregnancy loss will be of significant individual patient impact with respect to counselling and treatment. In addition, knowledge of specific genes that contribute to pregnancy loss could also be of importance in designing a diagnostic sequencing panel for patients with recurrent pregnancy loss, and also in understanding the biological pathways that can cause pregnancy loss.

Key words: genetic causes, pregnancy loss, euploid miscarriage, exome sequencing, chromosomal array, single nucleotide variation, copy number variant.

Introduction

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Miscarriage and recurrent pregnancy loss

Approximately 15–% of clinically recognised pregnancies end in pregnancy loss, with the majority occurring during the first trimester. Of these, 50–% are caused by chromosomal abnormalities such as aneuploidy (Hassold et al., 1980), and can be detected by conventional cytogenetic analysis. It is suggested that 86 % of these abnormalities are numerical, 6 % are structural abnormalities and 8 % are due to other genetic mechanisms, such as chromosomal mosaicism and molar pregnancies (Goddijn and Leschot, 2000).

Recurrent Miscarriage (RM) is defined by the Royal College of Obstetricians and Gynaecologists (RCOG) as at least three consecutive miscarriages before 24 weeks gestation (RCOG, 2011) and recurrent pregnancy loss (RPL) by the ESHRE November 2017 guidelines as the loss of two or more pregnancies (ESHRE, 2017). In addition to genetic aetiology, a spectrum of non-genetic causes of RPL have also been identified, including thrombophilic factors, endocrinological causes, immunological and immunogenetic causes, sperm DNA fragmentation, uterine malformations and lifestyle factors such as smoking (reviewed by Larsen et al. 2013).

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Cytogenetic and chromosomal microarray analysis

Traditionally, cytogenetic analysis of pregnancy tissue has been performed to identify genetic causes of RPL, and to indicate the need for further analysis of parental samples where there is the possibility of a balanced chromosome rearrangement (e.g. translocation) in one of the parents. It is important to identify any numeric chromosome errors, such as trisomy, monosomy or polyploidy, since these are causes of pregnancy loss which usually occur sporadically, and the likelihood of a successful pregnancy outcome is not negatively affected in subsequent pregnancies. Where there is a balanced translocation in one of the parents, genetic counselling is important as there is likely to be a recurrence risk in future pregnancies and pre-implantation genetic testing, chorionic villus sampling or amniocentesis can be used to detect an abnormality in the conceptus. However, for couples with a translocation, medical management (e.g. natural conception and observation) has been reviewed to have a higher live birth rate than IVF/PGD (Franssen et al., 2011, Hirshfeld-Cytron et al., 2011).

The most recent ESHRE guidelines for genetic analysis of products of conception (POC) give a conditional recommendation for genetic analysis but recommend that testing is carried out by array-comparative genomic hybridization (CGH) instead of traditional karyotyping (ESHRE, 2017). Conventional karyotype analysis identifies balanced and unbalanced chromosomal rearrangements and copy number variants (CNVs) to an approximately 5Mb resolution. Chromosomal microarray analysis can now identify unbalanced CNVs below 1Mb, with a resolution at the level of individual exons of genes in targeted regions of the genome (Miller et al., 2010). Microarray analysis is also less labour intensive as it is based on DNA analysis rather than cultured cells and has a higher success rate in poor quality tissue samples, however the quality of tissue will impact the success and failure rate of both

conventional karyotyping and array-CGH. Array-CGH has become the gold standard for genetic CNV analysis. It should, however, be noted that array--CGH may miss some balanced chromosomal rearrangements and may also fail to identify maternal cell contamination.

Other genetic causes

In the case of pregnancy loss, with an apparently euploid karyotype, there may be genetic aberrations causative of pregnancy loss that are not currently known or routinely assessed. These could include single-nucleotide variants (SNVs) that affect individual genes and are detectable by sequencing or small sub-microscopic aberrations that affect a cluster of genes and are detectable by microarray analysis. In the case of SNVs this is particularly important as many may follow a recessive or X-linked pattern of inheritance and therefore have a high recurrence risk. CNVs detected in cases of pregnancy loss may unmask a recessive mutation in a relevant gene or involve dosage sensitive genes, where loss or gain of copies affects the gene function. These regions may also represent benign CNVs seen frequently with no recorded effect on phenotype, although it remains possible that some may be involved in RPL. Evidence in humans and other species (Wilson et al., 2016) suggests that many genes are important in early development, and can lead to embryonic lethality when functionally "knocked out", resulting in pregnancy loss. More widespread genetic analysis of embryonic pregnancy loss may provide an opportunity to identify genes that are essential in early human development or where a lack of function leads to pregnancy loss.

Molar pregnancies

A molar pregnancy or Hydatidiform mole (HM) is an abnormal pregnancy, which has cystic degeneration of the chorionic villi, abnormal proliferation of the trophoblast and abnormal

development of the fetus. These can either be complete HM ~~(CHM)~~ or partial HM, distinguishable by the extent of trophoblast proliferation and presence of embryonic tissue. CHMs are usually diploid with all chromosomes of paternal origin. The majority arise from an anuclear ovum being fertilised by a haploid sperm and replicating its own chromosomes (uniparental paternal isodisomy), or rarely from an anuclear ovum fertilised by two sperm (uniparental paternal heterodisomy). HMs are mostly triploid with 23 chromosomes of maternal origin and 46 of paternal origin.

Whilst HMs are usually triploid and sporadic and therefore outside the scope of this review, a minority of molar pregnancies are diploid and biparental, usually being recurrent and familial. These may be caused by maternal autosomal recessive mutations in genes, such as NLR family, pyrin domain-containing 7 (*NLRP7*) and KHDC3-like protein, subcortical maternal complex member (*KHDC3L*), resulting in an abnormal epigenotype of imprinted loci. This results in abnormal gene expression, which causes abnormal placental trophoblast development and manifests as HM (Carey et al., 2015).

Whole exome sequencing

Advances in sequencing technology, including whole exome sequencing (WES) and whole genome sequencing (WGS), are increasingly providing the opportunity to detect genetic sequence variation and to characterise genetic mutations causing disease. WGS is the most extensive sequencing method and targets the entire genome, whereas WES targets the exome, which is the protein-coding region of the DNA. The exome makes up approximately 1% of the human genome, and it is estimated to contain 85% of the genetic mutations associated with disease (Choi et al., 2009). Generally, WES is the preferred method of

sequencing because it is cheaper than WGS and has a smaller, more manageable data set whilst still comprehensively covering the coding regions of DNA. WGS has the advantage of analysing and giving a comprehensive view of the whole genome and has the potential to detect large structural variants, insertions/ deletions, SNVs and copy number changes. However, we still understand relatively little about the non-coding regions of the genome.

Studies investigating the use of WES in structurally abnormal pregnancies, late pregnancy losses and developmental disorders (Wright et al., 2015, Shamseldin et al., 2018, Carss et al., 2014) have demonstrated the clinical application in these patients. However, very few WES studies have reported analysis in pregnancy loss or lethal genes which could contribute to RPL. The few studies using WES to look for genetic aberrations in RPL have also tended to represent only small patient cohorts. The ability to recognise and detect genetic mutations may have implications for routine genetic testing and clinical practice, especially when a pathogenic aberration is identified that can be reliably detected in future pregnancies.

Aims

There are several studies that have sought to identify genes causative of or associated with pregnancy loss, but the evidence has not yet been synthesised. We propose to review these studies and establish evidence of genetic causality of RPL, including reviewing appropriate methodologies. We will evaluate studies investigating Mendelian inheritance patterns, including autosomal recessive and dominant X-linked inheritance, and also *de novo* genetic causes, but we have excluded studies investigating more complex genetic associations, which have recently been systematically reviewed (Pereza et al., 2017).

Methods

Registration

This systematic review has been registered with PROSPERO (CRD42017073910).

Search

A systematic literature review to assess the studies investigating the genetic contribution to RPL was conducted in MEDLINE (1946 to May 2018) and Embase (1974 to May 2018) using Ovid (https://ovidsp.tx.ovid.com). The search terms used to identify pregnancy loss were "Spontaneous abortion", "miscarriage", "pregnancy loss" or "lethal", and the search terms to identify the genetic contributions are "exome", "human genome", "sequencing analysis", "sequencing", "copy number variation", "single nucleotide polymorphism", "microarray analysis" and "comparative genomic hybridisation". The search terms and corresponding Mesh terms are shown in Supplementary Table SI. Additional studies were also identified from references of selected studies.

Study selection

Studies were selected by two independent reviewers. Studies were first screened for eligibility using article titles and then by screening the study abstracts. Studies were included if they had pregnancy loss up to 20 weeks, but were not restricted if they also included some later losses, providing the genetic aberrations were defined. Studies were excluded if the genetic content included genes which were not lethal *in utero*, PGD studies, infertility studies, expression studies, aneuploidy with no recurrence risk, methodologies where there is no clinical relevance, and complex genetics. Both recurrent and sporadic

pregnancy loss were included. The full inclusion and exclusion criteria are presented in Supplementary Table SII.

Data extraction process

Data on publication date, country, study objective, sample, phenotype and gestation, methods and analysis, study outcome and quality scores were extracted. Data extraction was checked by a second reviewer. Each of the identified genes were found in Online Mendelian Inheritance in Man (OMIM) and the Mendelian Inheritance in Man (MIM) number, Gene name, gene function, associated disease/phenotype and cytogenetic location were ascertained.

Quality assessment

The quality of each study was assessed using a modified Newcastle-Ottawa scale (Supplementary Table SIII). Each study was scored out of 12 and was judged on the sample size, inclusion/exclusion criteria, the genetic analysis method, statistical analysis, case definition, controls and comparability. The breakdown of each score is included in Supplementary Table SIV.

Results

A total of 50 studies were included in the review. The initial search of the Medline and Embase databases identified 3404 potentially relevant articles. After screening the titles and abstracts, 74 full texts were obtained for detailed review. A total of 30 full articles were excluded because they were either not related to pregnancy loss, were more than 20 weeks gestation, or contained no genetic content. Examination of the bibliographies and journal

indices generated six additional studies for the review. Figure 1 illustrates the study selection. The papers identified were categorized into three themes; WES studies, CNV studies and other studies related to pregnancy loss including recurrent molar pregnancies.

The 50 studies that met the inclusion and exclusion criteria were all published in English between 2009 and 2018. Out of the studies identified, 21 were from Europe, 14 were from North America, 13 were from Asia and there was one study each from South America and Africa.

WES

Thirteen studies were identified (Table I) which used WES to identify SNVs in families with multiple pregnancy losses or a combination of pregnancy losses and terminations. Eight of these studies focused on a single couple only (Bondeson et al., 2017, Cristofoli et al., 2017, Dohrn et al., 2015, Filges et al., 2014, Rae et al., 2015, Shamseldin et al., 2013, Tsurusaki et al., 2014, Wilbe et al., 2015). Six studies used WES analysis of trios (Filges et al., 2014, Dohrn et al., 2015, Wilbe et al., 2015, Cristofoli et al., 2017, Bondeson et al., 2017, Qiao et al., 2016).

Studies using WES identified variants in genes from both fetal and parental samples, thus allowing for the inheritance to be identified. One study identified compound heterozygous mutations in kinesin family member 14 (*KIF14*) in a family with unexplained euploid miscarriages (Filges et al., 2014). The other studies included pregnancies terminated for a fetal abnormality including; a homozygous missense mutation in endothelin-converting enzyme-like 1 (*ECEL1*) from a consanguineous couple with pregnancies terminated due to

Arthrogryposis Multiplex Congenita (Dohrn et al., 2015); a novel homozygous mutation in the muscle, skeletal, receptor tyrosine kinase (*MuSK*) gene in a non-consanguineous couple with a history of fetal akinesia deformation sequence (FADS) (Wilbe et al., 2015); compound heterozygous mutations in SCL/TAL1-interrupting locus (*STIL*) from a non-consanguineous couple with fetal microcephaly (Cristofoli et al., 2017), a homozygous nonsense mutation in centrosomal protein, 55-KD (*CEP55*) in a non-consanguineous family with two~~2~~ fetuses with Meckel-like syndrome (Bondeson et al., 2017) and compound heterozygous mutations in intraflagellar transport 122 (*IFT122*) in a couple experiencing both RPL and later losses with scan abnormalities (Tsurusaki et al., 2014).

Two studies (Rae et al., 2015, Shamseldin et al., 2013) identified pathogenic variants by WES of fetuses affected with hydrops fetalis. The first identified pathogenic variant in the gene forkhead box P3 (*FOXP3*) was from a non-consanguineous couple whom had multiple male pregnancy terminations. *FOXP3* is an X-linked gene which is known to cause fetal akinesia syndrome (Rae et al., 2015). The second identified novel mutation in the gene cholinergic receptor, nicotinic, alpha polypeptide 1 (*CHRNA1*) was identified in a consanguineous couple (Shamseldin et al., 2013). Autosomal recessive mutations in this gene are also known to cause fetal akinesia.

A single study identified a homozygous missense variant in nucleolar protein 14 (*NOP14*) in pregnancy loss material from two consanguineous Iranian couples experiencing RPL. WES was completed on fetal tissue samples and the heterozygous copies of the variant were confirmed in the parents using Sanger sequencing (Suzuki et al., 2018).

Studies also used WES in larger cohorts. One study (Shamseldin et al., 2015) looked at consanguineous couples with two or more pregnancies diagnosed with non-immune hydrops fetalis (NIHF). Seven pathogenic variants previously known to cause NIHF (Shamseldin et al., 2015) were identified from 24 consanguineous couples with lethal NIHF.

Two Studies (Ellard et al., 2015, Qiao et al., 2016), analysed non-consanguineous couples with RPL. Variants in RNA export mediator (*GLE1*), ryanodine receptor 1 (*RYR1*) and DYNEIN, cytoplasmic 2, heavy chain 1 (*DYNC2H1*) were identified using WES of parental samples only (Ellard et al., 2015). Compound heterozygous variants were also identified in *DYNC2HI* and 15-lipoxygenase, reticulocyte arachidonate (*ALOX15*) in seven euploid pregnancy losses from four families (Qiao et al., 2016).

The final study used a slightly different approach and analysed a panel of 234 pre-selected RPL candidate genes from women affected by RPL. Using WES and bioinformatic filtering of non-synonymous sequence variants, 27 variants were identified from the previously selected genes (Quintero-Ronderos et al., 2017). The genes in which variants were identified in the described sequencing studies are detailed in Table II. However, genes from Quintero-Ronderos et al. 2017 have been excluded because they were from a pre-selected gene panel and therefore would introduce bias.

CNVs

Thirteen studies and one meta-analysis (Bagheri et al., 2015) (Table III), were identified which looked for CNVs in fetal tissue, parental samples or both by chromosomal microarray analysis. Three different microarray platforms were used for analysis, either single nucleotide polymorphism (SNP) array, oligonucleotide (oligo) array or bacterial artificial chromosome (BAC) array.

Six studies reported CNVs in pregnancy loss (Zhang et al., 2009, Viaggi et al., 2013, Levy et al., 2014, Zhang et al., 2016, Donaghue et al., 2017, Zhou et al., 2016), four studies in RPL (Rajcan-Separovic et al., 2010a, Nagirnaja et al., 2014, Karim et al., 2017, Robberecht et al., 2012) and three studies with a mixture of both pregnancy loss and RPL (Wang et al., 2017, Warren et al., 2009, Rajcan-Separovic et al., 2010b). Seven of the studies included parental samples and therefore the inheritance of reported CNVs was determined. Six of the studies did not include parental samples, and therefore the inheritance pattern of the CNVs reported in these studies could not be determined.

The pregnancy losses reported were pregnancies of varying gestational age, with the majority of pregnancy losses at less than 20 weeks. In three studies (Rajcan-Separovic et al., 2010a, Robberecht et al., 2012, Viaggi et al., 2013), all pregnancy losses tested were less than 12 weeks gestation. Two papers (Rajcan-Separovic et al., 2010b, Robberecht et al., 2012) also identified pregnancies with developmental abnormalities and used hystero-embryoscopy to allow morphological examination of the fetus *in utero* prior to genetic analysis.

Of the studies which determined the inheritance of the CNVs, there were 30 *de novo*, and 43 inherited CNVs (Levy et al., 2014, Rajcan-Separovic et al., 2010a, Rajcan-Separovic et al., 2010b, Robberecht et al., 2012, Wang et al., 2017, Warren et al., 2009). In general, the studies showed a 2.2 % - 13 % detection rate (DR) of pathogenic CNVs (Donaghue et al., 2017, Levy et al., 2014, Wang et al., 2017, Warren et al., 2009, Zhang et al., 2016, Zhang et al., 2009) plus a 0.9 % to 3.3 % DR of variants of unknown significance (VOUS) (Donaghue et al., 2017, Wang et al., 2017, Zhang et al., 2016, Qiao et al., 2016). An additional meta-analysis study (Bagheri et al., 2015) compared the characteristics and contributions of rare and common CNVs from four of the other studies by reclassifying CNVs according to the prevalence of healthy controls using Database of Genomic Variants (Bagheri et al., 2015, Rajcan-Separovic et al., 2010a, Rajcan-Separovic et al., 2010b, Robberecht et al., 2012, Viaggi et al., 2013). They concluded that common CNVs were specifically enriched in immunological pathways and rare CNVs were not, although the small number of rare CNVs may have hampered this conclusion. However, both rare and common CNVs could have a role in pregnancy loss, as rare CNVs have a two times higher gene density and contain more genes studied in mouse knockouts and common CNVs contain more genes in biological pathways relevant to pregnancy. The studies which identified VOUS were in accordance with each other and suggested the rate of 2-3 %.

Of particular interest is to find recurrent CNVs that are associated with pregnancy loss. Maisenbacher et al (Maisenbacher et al., 2017) determined the frequency of the 22q11.2 deletion in a large cohort of pregnancy loss samples using a SNP microarray. The 22q11.2 deletion was detected in 15 (0.07%) of 22451 POCs, with an overall incidence of 1/~1497.

They concluded that this was higher than the reported general population prevalence (1/4000- 1/6000). Likewise, Nagirnaja et al. (2014) identified CNV regions on chromosome 5 (5p13.3), disrupting the PDZ domain-containing 2 (*PDZD2*) and golgi phosphoprotein 3 (*GOLPH3*) genes. There was significant association with an increased risk of RPL. *PDZD2* and *GOLPH3* are predominately expressed in the placenta, suggesting a functional relevance, however neither of these genes haves previously been linked to placental function or pregnancy complications (Nagirnaja et al., 2014).

Recurrent molar pregnancies

Eleven studies (Table IV) were identified which evaluated the genetics of diploid and biparental recurrent HM (RHM) pregnancies. One study (Parry et al., 2011) identified biallelic mutations in chromosome 6 open reading frame 221 (*C6orf221*) in three consanguineous families with familial biparental HM. Three studies (Abdalla et al., 2012, Brown et al., 2013, Ulker et al., 2013) reported case studies of an individual consanguineous family, two non-consanguineous families and two consanguineous families with RHM. Autosomal recessive mutations were identified in the *NLRP7* gene and were considered to be responsible for the occurrence of HM. Deveault et al. investigated 13 women experiencing RHM, some with a family history of molar pregnancies and 11 *NLRP7* variants were identified (Deveault et al., 2009). Mutation analysis of the *NLRP7* gene in 35 women experiencing RPL with at least one HM revealed 17 different mutations (Qian et al., 2011). Qian et al. (2011) also suggested that one defective allele in *NLRP7* causes diploid androgenic moles and two defective alleles causes diploid biparental moles.

Two studies (Huang et al., 2013, Messaed et al., 2011) investigated cohorts of women to see whether mutations in the *NLRP7* gene could also be responsible for RPL without history of molar pregnancy. Messaed et al. (2011) investigated 135 women with either RPL or at least one HM and sequencing of *NLRP7* exons identified two patients with RPL to have *NLRP7* mutations. Huang et al. (2013) also showed significant association between RPL and *NLRP7* polymorphisms. In contrast, two further studies (Andreasen et al., 2013, Manokhina et al., 2013) identified no disease-causing mutations in *NLRP7* in women with RPL and similarly Aghajanova et al. (Aghajanova et al., 2015) found no mutations in *NLRP7*, NLR family, pyrin domain-containing 2 (*NLRP2*) or KHDC3-like protein, subcortical maternal complex member (*KHDC3L*) (*C6orf221*).

Other genetic causes

Two studies (Seyedhassani et al., 2010a, Seyedhassani et al., 2010b) analysed and sequenced mitochondrial tDNA (**AUTHOR:** is tDNA correct here?) in 96 women with RPL. Four variants in threonine transfer RNA (tRNA) and one variant in proline tRNA were observed, but in some cases these were also observed in controls (Seyedhassani et al., 2010a), which calls into question the significance of these findings. Analysis of mitochondrial D-loop sequences showed a higher rate of point mutations in RPL patients than in controls. In total, 89 out of 153 variants were only identified in women with RPL and 22 of these mutations were considered to be significant (Seyedhassani et al., 2010b).

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X-chromosome inactivation occurs during early embryogenesis and has also been proposed to have an aetiological role in RPL. Skewed X-chromosome inactivation (XCI) status was compared between women with RPL and healthy controls. Extremely skewed XCI (defined

as >90 %) was identified in 17.7% of women with RPL compared to 1.6 % of extremely skewed XCI in controls (Bagislar et al., 2006).

Six further papers were identified that discussed specific genes and their contribution to pregnancy loss. Each paper (Bendroth-Asmussen et al., 2016, Bhuiyan et al., 2008, Lopez-Carrasco et al., 2013, McKie et al., 2014, Stouffs et al., 2011, Zhang et al., 2016) investigated an individual gene or genes. In a case study of a 30-year-old women with pregnancy loss from glycogen storage disease Type IV (GSD-IV), DNA extracted from placental tissue identified compound heterozygous mutations in glycogen branching enzyme (*GBE1*) (Bendroth-Asmussen et al., 2016).

Another case study, a consanguineous Arabian family with pregnancy losses, stillborn, fetal demise and two live children, had homozygosity mapping. This led to the screening of the human ether-a-go-go-related gene (*HERG*) gene in the live children, parents and stillborn. Homozygous nonsense mutations in *HERG* were identified in the child with polymorphic ventricular tachycardia and the same heterozygous mutation in the parents and unaffected child. Amniotic fluid cells from the stillborn child were also homozygous for the same *HERG* mutation (Bhuiyan et al., 2008).

Three rare homozygous RYANODINE RECEPTOR 1 (*RYR1*) variants were identified using genome-wide linkage studies and sequencing of *RYR1* coding exons. Initially a *RYR1* homozygous nonsense mutation was detected in two fetuses with fetal akinesia deformation sequence (FADS)/ lethal multiple pterygium syndrome (LMPS). The parents were both homozygous for the same mutation. When 66 further probands with FADS/ LMPS

phenotype were screened for germline *RYR1* mutations, two further potential homozygous mutations were detected (McKie et al., 2014).

In a larger study, 100 couples with at least three unexplained pregnancy losses had wingless-type MMTV integration site family, member 6 (*WNT6*) mutation analysis performed. *WNT6* has previously been shown to have an important role for stromal cell proliferation during decidualisation in mice. Four novel mutations were identified in the women with RPL but not in the male partners or healthy controls (Zhang et al., 2015), although there was no conclusive evidence for pathogenicity.

Ten aberrations were identified in MutS, E. coli, homolog of, 4 (*MSH4*), DNA methyltransferase 3-like protein (*DNMT3L*) and synaptonemal complex protein 3 (*SYCP3*) in 23 couples with RPL. Six of these aberrations were predicted to alter the amino acid sequence. All but one of these aberrations was considered a likely SNV. The mutation in the *SYCP3* gene was shown to have a 78 % likelihood of causing a deleterious effect on protein function due to an alteration in the amino acid sequence changing a non-polar isoleucine into a polar threonine (Stouffs et al., 2011). Another study (Lopez-Carrasco et al., 2013) targeted the two spindle checkpoint genes aurora kinase B (*AURKB*) and *SYCP3* in 102 patients with either RPL or spermiogram alterations. One heterozygous intronic deletion was identified in *SYCP3* with no *in silico* causative indication. Six aberrations were identified in *AURKB,* however a deletion and two nucleotide changes were considered to have no functional alteration or be frequent variants respectively. Three rare missense variants were identified in *AURKB*, with two of these variants found in a couple with pregnancy loss.

Discussion

In this systematic review we have identified 50 papers which investigated genetic contributions other than aneuploidy to pregnancy loss. The studies highlight some key areas, including identification of SNVs by WES, identification of CNVs by microarray analysis, and investigation of a group of genes associated with diploid and biparental recurrent molar pregnancies that are linked to pregnancy loss. Other genetic contributions, such as epigenetics and mitochondrial DNA (mtDNA), were also investigated in individual papers. There were also studies reporting sequencing of candidate genes already known to be associated with pregnancy loss with or without structural abnormalities.

We have summarised the current evidence below for each of these categories, and then discuss the implications of these findings both for future studies and for genetic investigation of couples experiencing RPL.

WES

Advances in next generation sequencing are vastly improving and enabling a molecular diagnosis for a range of disorders and clinical pathways. As the cost of WES decreases, the technology is becoming more widely used and clinically applicable. This review identified a number of studies (Table I) over the last 4 years which have used WES to look for as yet unidentified genetic causes of pregnancy loss. The majority of these studies looked at individual patients or couples with RPL, some of which showed ultrasound scan abnormalities during the pregnancy (Bondeson et al., 2017, Cristofoli et al., 2017, Wilbe et al., 2015, Tsurusaki et al., 2014). More recently a small number of studies have been published studying larger cohorts of patients and exploring possible strategies for genetic

investigation of these patients (Ellard et al., 2015, Qiao et al., 2016, Shamseldin et al., 2015). This review included studies where patients suffered multiple pregnancy losses with phenotypic findings in all or some of their pregnancy losses. This included ultrasound scan abnormalities and post-mortem findings, and in some cases, where patients opted for termination of pregnancy. These were thought to be important to include because there could be a range of phenotypic effects caused by a genetic abnormality in a lethal gene, which could include abnormalities and late fetal death in some pregnancies, but pregnancy loss in others.

Bioinformatic filtering is required when studying the whole exome in order to provide a more manageable approach to interpretation of the data. In most of these studies 'trios' of patients were sequenced, and bioinformatic modelling of inheritance patterns was used to limit the number of variants identified. In most cases patterns of autosomal recessive inheritance (or X-linked recessive in male fetal losses) were modelled to look for variants. As might be expected, very often the couples investigated were consanguineous or possibly from populations isolated geographically. An alternative autozygosity mapping approach was used by Shamseldin et al. to restrict the genes that were analysed by WES (Shamseldin et al., 2013, Shamseldin et al., 2015) and a 'proof of principle' study (Ellard et al., 2015) developed a technique to identify autosomal recessive lethal disorders using WES in couples with RPL.

It is important to note that where autosomal recessive mutations are identified as a cause of pregnancy loss, this will guide counselling and treatment options for the couple as there is a

1:4 recurrence risk in future pregnancies, and prenatal diagnosis or PGD would be available to the couple.

Interestingly, genes that were identified from these WES studies are associated with processes that have an early role in developmental biology and are essential in embryogenesis. Some key processes include centrosome integrity, anti-inflammatory/ immune responses, proliferation and maintenance of epithelial cells, maintenance and development of collagen and muscle tissues, and blood coagulation. The majority of WES studies focused on individual families. Therefore the genes detected are limited to preselected cases and it is not possible to group them together for a meta-analysis to ascertain the detection rates.

Immune cells present early during pregnancy, especially during implantation where the maternal immune system has to tolerate the implanting embryo. The immune response during implantation is not currently well understood. However, the maternal immunity shifts from cell-mediated immunity to humoral (antibody mediated) immunity to protect the embryo from rejection. Aberrations in several genes, *ALOX15 (Qiao et al., 2016)*, complement component receptor 1 (*CR1*) (Quintero-Ronderos et al., 2017), *FOXP3* (Rae et al., 2015) and TOLL-LIKE RECEPTOR 3 (*TLR3*) (Filges et al., 2014) were identified and are known to be involved in inflammatory and immune defences. Mutations in these genes could be causing defects resulting in early pregnancy loss because the immune response is rejecting the embryo.

During embryogenesis, cells differentiate and proliferate. Potentially causative mutations were identified in FMS-related tyrosine kinase 1 (*FLT1*) (Quintero-Ronderos et al., 2017), leukemia inhibitory factor receptor (*LIFR*) (Quintero-Ronderos et al., 2017) and ubinuclein 1 (*UBN1*) (Shamseldin et al., 2015) genes involved in cell differentiation and proliferation. Mutations in the two genes trophinin (*TRO*) and cadherin 11 (*CHD11*) were both identified (Quintero-Ronderos et al., 2017) and are involved in cell adhesion. As cell differentiation, cell proliferation and cell adhesion are an important part of fetal growth during pregnancy, disruption in these genes could cause the pregnancy to fail.

Mutations in genes involved in tissue formation were also identified. In particular, cadherin 1 (*CDH1*) (Quintero-Ronderos et al., 2017) and frizzled, drosophila, homolog of, 6 (*FZD6*) (Shamseldin et al., 2015) are specifically involved in cell adhesion, matrix metalloproteinase 10 (*MMP10*) and matrix metalloproteinase 9 (*MMP9*) (Quintero-Ronderos et al., 2017) for extracellular remodelling, and *MuSK* (Wilbe et al., 2015) and myomesin 1 (*MYOM1*) (Shamseldin et al., 2015) for formation of neuromuscular junctions and striated muscle.

During pregnancy, blood passes through the placenta for the exchange of gases, nutrients, electrolytes and waste products between the mother and fetus. Mutations in three genes, coagulation factor V (*F5*), fibrinogen, A alpha polypeptide (*FGA*) and thrombomodulin (*THBD*) (Quintero-Ronderos et al., 2017), were identified. These are involved in the coagulation pathway. The flow of blood is necessary for the fetus to grow and any disruption causing the blood to clot could result in loss of the pregnancy.

564 In summary, WES of POC or fetal DNA and parental DNA is a promising method to identify
565 variants in genes which might be responsible for RPL and/ or fetal abnormalities. Where
566 aberrations are inherited from the parents, a genetic diagnosis may provide invaluable
567 information for preimplantation screening or prenatal diagnosis in future pregnancies.
568 However, studies with larger unbiased cohorts are needed to conclusively determine
569 detection rates and the clinical utility of WES in this group of patients.
570
571 Chromosomal microarray analysis
572 In some cases, CNVs either as gains or losses may be responsible for pregnancy loss of a
573 fetus with an apparently normal karyotype. CNVs, both rare and common, may be impacting
574 pregnancy-related genes or pathways, resulting in pregnancy loss. These may involve single
575 genes or clusters of genes which are deleted, duplicated or disrupted.
576
577 Studies identified by our systematic review are summarised in Table III. Due to the diverse
578 approaches taken, the studies are difficult to compare collectively. Cohorts reported
579 sporadic pregnancy loss and RPL, different gestations and different methods of analysis.
580 Some studies (Bagheri et al., 2015, Rajcan-Separovic et al., 2010a, Rajcan-Separovic et al.,
581 2010b, Warren et al., 2009, Levy et al., 2014, Robberecht et al., 2012, Wang et al., 2017)
582 analysed both fetal tissue and parental DNA concurrently (i.e. a trio) to identify whether
583 CNVs were *de novo* or inherited. This is important in assessing both the likely pathogenicity
584 of the finding and the associated recurrence risk. Where the CNV is also detected in a
585 parent it is less likely to be causative of a pregnancy loss in isolation. It is possible that
586 inherited CNVs could still cause RPL where the CNV co-occurs with an autosomal recessive
587 gene mutation (SNV) on the other allele or where genes present within the CNV are relevant

588 to genomic imprinting or embryonic/ placental growth (Rajcan-Separovic et al., 2010a,
589 Rajcan-Separovic et al., 2010b).
590
591 Relatively little is known about the genes and pathways involved in pregnancy loss, and
592 therefore many CNVs identified will be classed as having uncertain clinical significance. One
593 study analysed CNVs in parents experiencing idiopathic RPL using functional enrichment
594 analysis, identifying biological pathways that were significantly over-represented, such as
595 antigen binding and immune signalling (Karim et al., 2017, Nagirnaja et al., 2014).
596 Enrichment was identified in genes associated with immunoregulatory interactions at the
597 feto-maternal interface and impaired immune signalling (Nagirnaja et al., 2014).
598
599 Identification of pregnancies with developmental abnormalities using hystero-embryoscopy
600 enables genetic abnormalities to be compared with developmental abnormalities and
601 growth disorganisation of the embryo. CNVs identified where there is a developmental
602 abnormality present are more likely to indicate genes important in early development. In
603 addition to evaluating a genetic cause for pregnancy loss, such studies can provide an
604 opportunity to identify and evaluate the function of the genes. Where variants are identified
605 in genes, through analysis of an enriched cohort~~, such as this~~ with developmental
606 abnormalities, it is easier to interpret their clinical significance.
607
608 Several studies explored the possibility of uniparental disomy (UPD) and looked for regions
609 of Loss of heterozygosity in euploid embryos (Levy et al., 2014, Robberecht et al., 2012,
610 Wang et al., 2017). The pathological relevance of UPD is difficult to evaluate as not all
611 platforms are capable of detecting UPD (eg. Oligo BAC array) and therefore are difficult to

compare. Pregnancy loss could be due to UPD resulting in unmasking of an underlying lethal recessive disease gene(s) or imprinted genes.

CNVs were identified in the highly imprinted region 11p15.5. This region is abundant with imprinted genes and has an important role in the maternal-fetal exchange. Aberrant methylation or duplication of imprinted genes in this region could cause pregnancy loss (Zhang et al., 2016).

Recurrent molar pregnancies

Although the majority of HM are sporadic, a small minority are recurrent and/-or familial. A number of studies looked at the role of genes including *NLRP7*, C*6orf221* (*KHDC3L*) and *NLRP2* in pregnancy loss manifesting as recurrent molar pregnancy. In the cases reviewed, the HM are euploid, and are instead caused by autosomal recessive mutations in genes which code for the cell machinery that labels the parental origin of the two sets of chromosomes.

It is thought that *NLRP7* and *C6orf221* are components of an oocyte complex that forms during oogenesis and determines the epigenetic status of the oocyte genome by inactivating genes. It is likely that mutations in *NLRP7* cause HM by impairing the normal imprinting process causing maternal genes to be expressed when they should not be.

Studies have explored the role of *NLRP2*, NLR family, pyrin domain-containing 5 (*NLRP5*), *NLRP7* and *C6orf221* in other forms of pregnancy loss such as partial moles, RPL, stillbirth, infertility and multi-locus imprinting disturbance (Aghajanova et al., 2015, Andreasen et al.,

2013, Huang et al., 2013, Manokhina et al., 2013, Messaed et al., 2011, Docherty et al., 2015). These have shown conflicting results, many showing no evidence of *NLRP7*, *NLRP2* and *C6orf221* mutations in women with RPL (Aghajanova et al., 2015, Andreasen et al., 2013, Manokhina et al., 2013).

Evidence from several papers suggests that genes involved in oocyte development, maturation and epigenetic reprogramming are likely to be important in a subset of pregnancy losses. One of the most studied epigenetic modifications is DNA methylation. DNA methylation is implicated in the regulation of imprinting and the expression of imprinted genes is thought to be important for the development and physiology of the placenta (Frost and Moore, 2010). Aberrant DNA methylation of several imprinted loci (H19, imprinted maternally expressed noncoding transcript (*H19*), long QT intronic transcript 1 (*LIT1*) and small nuclear ribonucleoprotein polypeptide N (*SNRPN*)) was demonstrated in pregnancy losses, with increasing methylation of these genes showing a positive correlation with pregnancy loss. It is possible that inappropriate DNA methylation may either be a contributing factor or consequence of the defect that led to pregnancy loss (Zheng et al., 2013). It also remains to be investigated as to whether there are wider epigenetic defects at other loci. Zheng et al. (2013) propose a multifactorial threshold model for pregnancy loss where additional genetic and environmental factors may also play a role.

Other genetic causes

Mitochondria have been hypothesised to have an important role in development. They predominantly regulate the production of ATP, used to regulate cellular metabolism.

Processes such as cell proliferation and development require high energy giving the mitochondria an important role during pregnancy. Seyedhssani et al. (Seyedhassani et al., 2010a, Seyedhassani et al., 2010b) have identified mutations in mtDNA in women with RPL (Seyedhassani et al., 2010b). Furthermore a significant number of mutations were identified in the D-loop of mtDNA. The D-loop contains essential elements for mtDNA transcription and disruption could affect the transcription or translation of mtDNA, in turn compromising embryonic development or causing pregnancy loss.

It is hypothesised that skewed XCI could be involved in the pathogenesis of RPL. Bagislar and colleagues (Bagislar et al., 2006) demonstrated extremely skewed XCI in 17.7 % of patients with RPL. It is suggested that skewed XCI could expose X-linked variants that are lethal in the hemi-zygous state. In addition, a more recent review (Sui et al., 2015) included 12 case-control studies on skewed XCI with or without RPL. In patients with RPL, skewed XCI was significantly higher, although the significance drops with fewer losses and for less extreme skewing. Although the association between RPL and skewed XCI is unclear, two mechanisms have been proposed. Firstly, if a female carrier with a recessive lethal X-linked genetic mutation and skewed XCI has a male fetus who inherits the X-linked genetic mutation, it could lead to pregnancy loss. Secondly, an X-linked genetic mutation could cause follicular atresia and an increase in aneuploid embryos resulting in pregnancy loss (Sui et al., 2015).

Six papers (Bendroth-Asmussen et al., 2016, McKie et al., 2014, Stouffs et al., 2011, Zhang et al., 2016, Bhuiyan et al., 2008, Lopez-Carrasco et al., 2013) describe targeted sequence analysis of specific candidate genes (*GBE1*, *RYR1*, *WNT6*, *DNMT3L*, SYCP3, *MSH4, HERG* and AURKB) in either an individual case of pregnancy loss (Bendroth-Asmussen et al., 2016,

Bhuiyan et al., 2008) or in patient cohorts (McKie et al., 2014, Stouffs et al., 2011, Zhang et al., 2016, Lopez-Carrasco et al., 2013). This targeting was informed by factors including histopathological examination of placental tissue observed in fetal arrhythmia, scan findings and functional prediction of gene pathways.

Limitations of current evidence

This review was completed in a systematic manner by two independent reviewers making it reproducible. The limitation of this study, however, is the quality of the studies published to date. Each study was scored according to our modified Newcastle-Ottawa scale (Supplementary Table SIV) with a few of the studies being of poor quality and scoring as little as 3 or 4 on our scale.

The most common limitations in these studies related to the small size of the studied cohorts, with several focusing on a single family, and many of the studies lacking information on control populations or statistical analysis. Work on small groups, and in particular a single family, may detect genetic abnormalities that have occurred in isolation or are very rare. In many cases this results in identification of variants in unique candidate genes with no definitive causal effect. Therefore larger cohorts are needed to replicate these findings and to determine how relevant these findings are to other couples with RPL.

There was also limited availability of functional data in many of the studies. A few studies supplemented their cases with information on scan abnormalities or post-mortem abnormalities detected in cases of losses and hystero-embryoscopy to correlate genetic

findings with findings in the embryo. The studies were also difficult to compare and collate as there were multiple variations in the cohorts studied and the methods of analysis.

Conclusion

It is evident that there are many genetic and environmental factors that result in a successful pregnancy and a disruption in any of these could contribute to pregnancy loss. From the genetic perspective this includes both clearly pathogenic genetic causes, such as sporadic aneuploidy and translocations, and other potential genetic causes such as smaller CNVs and mutations in genes important in early fetal development. In addition, there are likely to be complex genetic contributions, such as multi-factorial inheritance, and changes in methylation (epigenetics) and mitochondrial function, which could be contributing to pregnancy loss. These more complex genetic mechanisms may be influenced by environmental factors, such as diet, medication, pollutants and lifestyle, which could provide a cumulative effect resulting in pregnancy loss.

The papers we have identified have demonstrated that monogenic aetiologies could contribute to a proportion of pregnancy losses. However, as most studies have been carried out in highly selected families or small cohorts, additional studies are required to further assess if this technology is generalisable to more couples experiencing RPL.

It is plausible that cases of pregnancy loss (particularly in RPL) may have causative mutations not detectable with routine cytogenetic analysis or fetal scans, but are detectable by WES. Although WES is not currently recommended for routine diagnostic use for pregnancy losses, the identification of genes associated with pregnancy loss will be of significant

731 individual patient impact with respect to treatment and availability of PGD. If monogenetic
732 etiologies of RPL and the overall prevalence of monogenetic causes of pregnancy loss are
733 better elucidated through larger, well-designed studies, the identification of non-aneuploid
734 causes of RPL could be of significant patient impact.
735
736 Knowledge of specific genes that contribute to pregnancy loss could also be of importance
737 in understanding the biological pathways that can cause pregnancy loss. However, much
738 larger and more comparable cohort studies are required in all of these areas to determine
739 causality of candidate genes and to dissect out these effects, as at present many of these
740 findings are of uncertain clinical significance. Functional analysis, such as embryoscopy
741 studies and *in vivo* animal modelling, may assist in further assessment of the mutation effect
742 on early embryonic development.
743
744 RPL is a complex problem influenced by many different aetiologies. Currently, with the
745 exception of aneuploidy and other chromosomal abnormalities, routine investigation for the
746 genetic contributions causing pregnancy loss is limited. With increased knowledge of
747 additional non-aneuploid contributions to RPL, additional genetic testing recommendations
748 may be made in the future to couples experiencing RPL. These would have implications for
749 diagnosis and recurrence risks.
750
751 **Authors' roles**
752 EC- Study search, study selection, data extraction, quality assessment and writing.
753 SH- Data extraction, quality assessment and editing
754 PS- Study design, critical appraisal of manuscript

NM- Critical appraisal of manuscript and editing

AC- Study design and critical appraisal of manuscript

SA- Supervision, study selection, writing and editing

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Conflict of interest

There are no conflicts of interest to declare.

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AUTHOR: please would you recheck journal style for the references and edit accordingly?

Thank you (e.g. upper/lower case, bold text).

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Figure legend

Figure 1 PRISM flow diagram for a systematic review of the potential genetic causes of miscarriage in euploid pregnancies.

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