Review Article

The male contribution to recurrent pregnancy loss

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Abstract: There are several known causes of recurrent pregnancy loss (RPL) in a couple, which include endocrine abnormalities, immunologic abnormalities, structural uterine abnormalities and karyotype abnormalities. The evaluation largely focuses on the female. The male contribution to RPL remains understudied. With the exception of the karyotype analysis, there is currently no other recommended testing for the male partner of a woman who has suffered multiple pregnancy losses. Chromosomal abnormalities are well defined causes of pregnancy losses in the literature. However, despite the fact that abnormal DNA fragmentation has been implicated in the pathogenesis of unexplained RPL, it is not routinely checked during the evaluation of RPL. This is likely due to the fact that abnormal DNA fragmentation is the end result of multiple different mechanisms including environmental exposures, varicoceles, gene alteration and epigenetic changes resulting in an inherent susceptibility to DNA damage? We are just beginning to scratch the surface of our understanding of the male contribution to RPL and more studies especially focusing on epigenetic modifications and gene alterations are needed.

Keywords: DNA fragmentation; epigenomics; recurrent miscarriages

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Introduction

Pregnancy loss is common and occurs in approximately 15–25% of clinical pregnancies. Recurrent pregnancy loss (RPL) is a distinct disorder defined as two or more failed clinical pregnancies (1). Fewer than 5% of women will experience two consecutive miscarriages and only 1% will experience three or more miscarriages (2). Our knowledge of the underlying etiologies behind RPL is still in flux. However, current evaluation of couples with RPL focuses on female factors including endocrine abnormalities such as thyroid disease, hyperprolactinemia and uncontrolled diabetes; uterine factors such as fibroids and Mullerian anomalies, acquired thrombophilia evaluation and karyotyping to evaluate for balanced translocations (3). The man, while important for conception, is investigated only with karyotype.

The semen analysis is generally not a part of the initial assessment of RPL due in part to its limitations as a functional test. However, sperm integrity is essential for sperm—egg interactions, fertilization and early embryonic development (4-6). In addition, paternally expressed genes modulate the proliferation and invasiveness of trophoblast cells and later placental proliferation (7-9). Despite some of the evidence of the effect of sperm on early embryogenesis and placental function, male factors contributory to RPL are largely unexplored.

Fifty percent of couples with RPL will receive the diagnosis of unexplained RPL (*Figure 1*) (3). This is a frustrating diagnosis that has both physical and psychological implications. There is suspicion that some of unexplained RPL is as a result of an underlying male

mechanism that is not currently understood. It is logical that since the male gamete contributes 50% of the genomic material to the embryo and placenta (10-12), the integrity of the sperm genome is essential for the initiation and maintenance of a successful pregnancy (13).

In this paper, we will discuss what is known about the male contribution to RPL. We will identify knowledge gaps and discuss the limitations of some of the findings in the literature. We will discuss some of the genes implicated in male infertility that appear to overlap with RPL, as well as some epigenetic modifications thought to be associated with RPL. Finally, we will attempt to provide a glimpse into the future and comment on what research must be done to answer some of the questions about the male contribution to RPL.

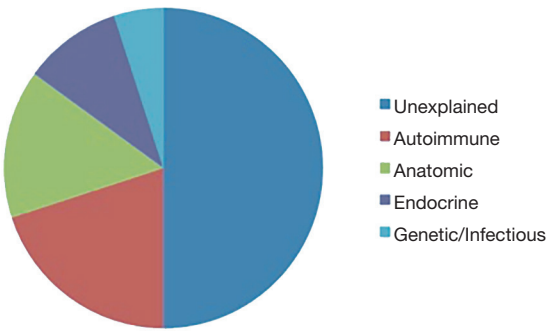

Figure 1 Etiologies of recurrent pregnancy loss (3).

What is known?

Structural chromosomal abnormalities

Structural chromosomal abnormalities imply a different arrangement of an appropriate number of chromosomes. For example, a part of one chromosome may be found attached to a different chromosome so that all the genetic material is present but not in the right place. This is referred to as a translocation. In this situation, it is possible to create unbalanced gametes, which often result in offspring that spontaneously abort (*Figure 2*). Balanced translocations consist of reciprocal or Robertsonian translocations or inversions. Translocations can occur de novo in an embryo or can be inherited from either parent. If the translocation is inherited, the carrier parent is often phenotypically normal.

Karyotyping of couples is part of the evaluation of RPL and karyotypic abnormalities are an established cause of RPL, whether the abnormality occurs in the male partner or the female partner. It is however often the last test that is obtained because of the low likelihood of an abnormal result. A review of cytogenetic findings in multiple published surveys of couples with two or more pregnancy losses observed an overall prevalence of major chromosomal abnormalities of 2.9%, which is five to six times higher than that of the general population. Approximately 50% of the abnormalities were balanced reciprocal translocations, 24% were Robertsonian translocations and 12% were sex chromosome mosaicisms in females; the rest consisted of

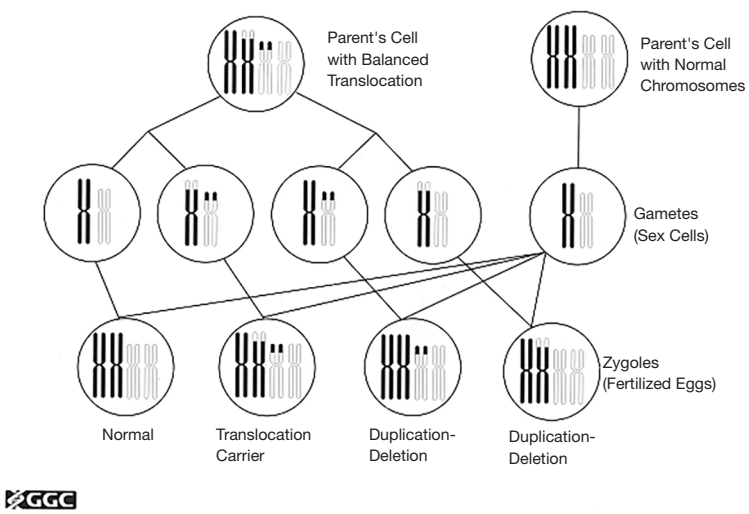

Figure 2 Reciprocal translocation. Illustration from Genetic Counseling Aids, 2nd Edition, Copyright 1989, permission for use granted by Greenwood Genetic Center.

inversions and other sporadic abnormalities. In every group of chromosome abnormalities in the parents, there was a female to male predominance with a 2:1 ratio (14). Thus, structural chromosomal abnormalities in the male partner contribute in only a small proportion of couples with RPL.

Sperm deoxyribonucleic acid fragmentation

DNA fragmentation is the separation or breakage of DNA strands into pieces. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and sperm chromatin structure assays (SCSA) have been used for the identification of significant DNA fragmentation in couples with infertility and RPL (15-18). Not only does the presence of significant sperm DNA fragmentation have profound implications on embryogenesis, prenatal and postnatal growth, it has also been proposed to be associated with congenital malformations and childhood cancers (19-21).

Fertilization of an oocyte with damaged spermatozoon may result in an increase in DNA damage in the resulting embryo genome, which could result in DNA errors at different levels of embryogenesis (22). This could manifest as either being lethal to a developing embryo or as childhood diseases if the errors are non-lethal (23,24). Furthermore, this type of damage could occur in embryos with a normal chromosome complement and therefore could contribute to unexplained RPL (25). Higher sperm DNA fragmentation in couples with RPL may have its origin in poor DNA packaging at chromatin remodeling during spermiogenesis, which could leave DNA more vulnerable to oxidative stress (26-29) and DNA nucleases (30,31).

Several studies have suggested an association between increased sperm DNA fragmentation and unexplained RPL (32-36). The study by Bareh *et al.* [2016] is especially intriguing because they only included normozoospermic male partners and nonetheless detected significantly higher levels of DNA fragmentation within the RPL group compared to controls (36). The paternal genome provides the centrosome in the first mitotic division after fertilization (37). Because the paternal genome is activated between the four- and eight-cell stage in human embryos, high DNA damage may have no effect on fertilization yet manifest in later stages of embryonic development (38,39).

Although ASRM does not recommend routine sperm DNA fragmentation testing in male partners of women with unexplained RPL, current evidence since that guideline was published suggests that this could provide a potential mechanism for a male contribution to unexplained RPL. Moreover, a variety of interventions have been demonstrated to decrease sperm DNA fragmentation. Varicoceles are a known cause of sperm DNA damage (40) and many reproductive urologists will evaluate for their presence in couples with RPL. Varicocelectomy decreases sperm DNA fragmentation (41). Indeed, a randomized controlled trial demonstrated higher rates of conception and lower rates of miscarriage in couples with RPL in whom the male underwent varicocele repair (42). Furthermore, Esteves *et al.* demonstrated the effectiveness of using testicular sperm for ICSI over ejaculated sperm during IVF as a strategy to overcome infertility in oligozoospermic men with high sperm DNA fragmentation (43).

Additional modifiable factors that have been associated with an increase in reactive oxygen species generation and abnormal sperm DNA fragmentation include alcohol (44), smoking (45) and some environmental toxins (45-47). Furthermore, we are just beginning to explore the possibility that some men could have an unrecognized inherent genetic predisposition that causes their spermatozoa DNA to become susceptible to fragmentation. This possibility is yet to be thoroughly investigated and would require refined genetic evaluations including assessing epigenetic modifications in the sperm genome.

Despite some of the evidence for DNA fragmentation as a potential etiology of RPL, there are some limitations for its use. The threshold for what is deemed as "abnormal" DNA fragmentation varies in the literature and until there is a standardized method of measuring DNA fragmentation, it may not be widely utilized in the evaluation of couples with unexplained RPL.

Y chromosome microdeletions

The presence of severe oligospermia or azoospermia on routine semen analysis warrants further investigation including evaluation for microdeletion of the azoospermic factor (AZF) on the Y chromosome (48). Prevalence of Y-chromosome microdeletion in severely oligospermic and azoospermic men is estimated to be 8–18% (49,50). Several investigators have studied the prevalence of Y-chromosome microdeletions in their populations of couples with RPL. Three studies have demonstrated a significantly higher prevalence of microdeletion in the Y chromosome in the RPL group compared to controls and this prevalence ranged from 16% to 82% (51-53), while other studies have not demonstrated a difference in the prevalence of

Y-chromosome microdeletion in the RPL group compared to the control group (54-56).

These studies on the association between Y-chromosome microdeletion and RPL are mixed in part due to the low prevalence of Y-chromosome microdeletion in the male infertile population coupled with the fact that these men are very rarely able to procreate, and almost exclusively require assisted reproductive technology (ART) for reproduction. In addition, it remains unknown how spermatozoa with a deletion influence fertilization rate and embryo quality. There are very few studies in the literature of the plausible mechanism by which AZF mutation can be implicated with miscarriages. Some of these studies implicate AZF region mutations with a meiotic defect, which may be associated with increased pregnancy loss (57-59). Another alternative explanation is that these Y-chromosome microdeletions are polymorphisms and due to the presence of palindromic areas, there are likely to be crossing over events with the X-chromosome yielding a genetic abnormality that could result in RPL (51). This is a knowledge gap that could be further investigated with animal models.

What is currently being explored?

Sperm aneuploidy

We are now beginning to understand that genetic and epigenetic contributions of sperm to early embryogenesis are extensive and have profound clinical implications. Fluorescent in situ hybridization (FISH) technology is the primary method used to study sperm chromosomes and detect aneuploidy. Sperm aneuploidy has been detected at an increased rate in male partners of women with RPL compared to controls in several studies (35,60-63). Although the data has shown increased rates of sex chromosome disomy in sperm from the male partner in couples with RPL, cytogenetic analysis of products of conception from couples with RPL does not reveal an increased rate of sex chromosome aneuploidy. This might suggest that cytogenetically abnormal sperm might be selected against during the process of fertilization (64,65). ASRM does not recommend routine sperm aneuploidy testing in couples with unexplained RPL (3). They cited the study by Stephenson *et al.* which demonstrated that over half of miscarriages in couples with RPL were euploid (54% *vs.* 46%) (64). However, one limitation of this study is the fact that they looked at a heterogeneous group with recurrent miscarriage and didn't limit to idiopathic cases.

Moreover, the 2015 ASRM practice guideline for evaluation of the infertile male suggests that patients with RPL may benefit from screening for sperm aneuploidy (48). This differing view may be due in part to uncertainty surrounding the prognostic value of FISH regarding the final progeny as well as cost considerations (66). Furthermore, most FISH studies focus on a small number of chromosomes, typically those associated with aneuploidies compatible with life, namely 13, 18, 21, X and Y (67). What is unknown is whether this limited FISH panel is sufficient for evaluation of couples with RPL or even if expanded panels would provide more information. In addition, Neusser *et al.* posited that in RPL, chromosomes 1, 2, 6, 15, 16 and 21 are more relevant targets for sperm aneuploidy testing with chromosome 16 being the most promising diagnostic target (68). For these reasons, some authors suggest that until more in-depth studies are performed to explore this relationship, men with RPL should be screened for sperm aneuploidy and also referred to genetic counselors (69). At present, no intervention is known to decrease sperm aneuploidy but preimplantation genetic screening (PGS) can be used to select for euploid embryos during IVF.

Methylenetetrahydrofolate reductase (MTHFR) polymorphisms

MTHFR enzyme plays an important role in catalyzing the conversion of 5,10-methylenetetraydrofolate into 5-methylenetetrahydrofolate, which provides the single-carbon for homocysteine in methionine synthesis (70,71). There have been several studies that have evaluated the association between polymorphisms in MTHFR reductase activity and unexplained RPL. The results of these epidemiologic studies have been inconsistent in the literature. Of the 40 different genetic polymorphisms of MTHFR, C677T variant is the most studied and thought to be the most clinically relevant. A recent meta-analysis of 29 articles demonstrated a significant association between the MTHFR C677T polymorphism and a susceptibility to RPL in women (72). In addition, a pooled meta-analysis of 57 articles also demonstrated that both maternal and paternal MTHFR gene C677T and A1298C variants are associated with RPL. They also observed a significant association between fetal MTHFR A1298C polymorphism and RPL, but no association with C677T (73). Due to the inconsistent literature on this association, a general consensus has not been determined on the impact of paternal MTHFR

polymorphisms on RPL.

Annexin A5 M2 haplotype

In 2007, a hereditary factor for RPL was suggested (74). This factor, called the M2 haplotype, comprises four consecutive nucleotide substitutions in the core promoter of the annexin A5 (ANXA5) gene and results in reduced expression levels of ANXA5 in placenta. ANXA5 is a member of the annexin protein family. It is ubiquitously expressed in perfused ductal organs and most abundantly present at the apical surfaces of the syncytiotrophoblast covering placenta villi (75). ANXA5 has potent anticoagulant properties that have been extensively studied both *in vitro* and *in vivo* (76,77). ANXA5 is crucial for the dynamics of membrane repair within the syncytiotrophoblast and reduced expression results in various thrombophilia-related pathologies of pregnancy such as preeclampsia, fetal growth restriction and RPL (75).

Abortion risk of women carrying the M2 haplotype for ANXA5 has been demonstrated to be over 2-fold higher than the general population (74). However, the mechanism by which this occurs is not known. It is suggested that the most likely explanation is a reduced expression of ANXA5 in placenta. It has also recently been demonstrated that the genetic frequency of paternal M2 carriage is significantly higher in couples with RPL than in fertile controls in the German population and its effects occur distinctly between the 10th and 15th week of gestation (75,78). Association between Annexin A5 M2 haplotype polymorphism and RPL has been replicated in other populations including Italian, Bulgarian, Japanese and Malaysian but the mechanism by which it impacts pregnancy loss needs to be further elucidated (78-81). Genotypic evaluation of embryonic tissue obtained from pregnancy loss may be relevant in further understanding the impact of this haplotype.

Ubiquitin-specific protease (USP26) gene alterations

In recent years, increased attention has been paid to genetic causes of male infertility. In addition to Y-chromosome microdeletion and mutation of some autosomal genes, X-chromosome genes have also been found to be closely related to male fertility; however, their underlying molecular mechanisms are still largely unknown (82,83). Nishimune and Tanaka [2006] observed many genes on the X-chromosome that are related to male infertility. The ubiquitin-specific protease 26 was first identified from a screen of X-linked genes involved in spermatogenesis by Wang *et al.* [2001]. USP26 belongs to a family of deubiquitinating enzymes, which play an important role in several biological processes such as control of growth, differentiation, oncogenesis and genome integrity (84,85). These enzymes might be involved in the removal of histones, regulation of cell turnover during meiosis, germ cell apoptosis, and proliferation and differentiation of spermatogonial stem cells during spermatogenesis (86).

In a recent study of 166 infertile men with non-obstructive azoospermia, 72 male partners of couples with RPL and 60 fertile controls, the authors demonstrated that total frequency of mutation in three common haplotypes of the USP26 gene in the study population was significantly higher in the infertile group and RPL group compared to the fertile controls. The authors concluded that in their population of Iranian men, alterations to the USP26 could impact fertility outcomes. Mutations may lead to an increase in histone levels in sperm DNA and consequently increased sperm DNA damage (86). Further studies are required to examine this association, which could potentially be applicable to men with idiopathic RPL.

Telomere length

Telomeres have specialized function in maintaining chromosome integrity and in germ cells, are thought to aid in meiotic recombination and pairing of homologous chromosomes. Telomere shortening in somatic cells results in telomeres losing their capping ability at the end of chromosomes, resulting in nonreciprocal translocations, chromosomal instability, deletions, aneuploidy and DNA damage (87). Liu *et al.* reported that shortened telomere length in male mice resulted in apoptosis, decreased recombination and meiotic arrest, while in females shortened telomeres led to impaired embryonic viability and fetal development (88). Telomeres are hypothesized to be one of the first structures in the sperm nucleus that respond to oocyte signals for male pronucleus development at fertilization (89).

Thilagavathi *et al.* [2013] therefore hypothesized that if telomeres are known to play a significant role in various disorders, they might also play a role at the level of the sperm and ova genome in unexplained RPL. Their study involved analyses of telomere length by real time qPCR of leukocytes obtained from 25 couples who experienced unexplained RPL and 20 fertile controls. The authors discovered that the relative leukocyte mean telomere

length in both men and women with unexplained RPL was significantly lower when compared to controls. This was an interesting finding and led the authors to conclude that shortened telomeres might play a role in unexplained RPL. However, this would need to be further substantiated by analyzing telomere lengths at the level of germ cells (90).

The era of sperm epigenetics

A growing area of research in infertility is the role of epigenetics in male fertility. Epigenetics refer to non-coding areas in the genome that do not alter the basic DNA sequence but play a regulatory role. Modifications to the epigenome can occur via several mechanisms such as methylation, micro-RNAs and histone modification (91,92). The sperm epigenetic profile might provide a historical information about the entire process of spermatogenesis. During maturation of sperm, about 90% of histones are replaced by protamines, and this allows for more efficient packaging of compacted chromatin and also protects the sperm from oxidative damage. Any modification to this process would impact the DNA integrity in sperm and render it susceptible to DNA damage (93).

For this reason, the sperm protamine 1 to protamine 2 mRNA ratio has been extensively evaluated as a parameter of sperm functionality and abnormal ratios have been suggested to be implicated in male infertility (94-96). It has also recently been demonstrated to be a prognostic indicator of IVF/ICSI outcomes (96). Furthermore, a recent study of 25 male partners of women who experienced unexplained RPL, 32 healthy sperm donors and 107 infertile cohort demonstrated significant differences between the RPL group and the healthy donors in protamine-1, protamine-2 mRNA levels as well as the protamine mRNA ratios (97). In particular, the authors discovered that spermatozoa from male partners of women with unexplained RPL contained significantly higher protamine-1 and protamine-2 and the protamine mRNA ratio was lower in the case group. The authors suggest that not only are protamines important for fertilization, they may play an additional role in early embryogenesis; albeit through an uncertain mechanism (97). Protamines warrant further investigation as its mechanism of impact on male infertility appears to dovetail with an impact on pregnancy loss.

MicroRNAs are also non-protein coding RNAs that induce post-transcriptional gene silencing and mediate translational repression (98). MicroRNAs are believed to regulate almost a third of the human genome (99). It has been suggested that single nucleotide polymorphisms (SNP) in microRNA sequences can potentially affect their regulatory function (99) and some studies have demonstrated an association with RPL (100,101). A recent comparison study of couples with unexplained RPL compared to proven controls demonstrated differences in parental microRNA polymorphisms between the cases and the controls. This was the first study to implicate male microRNAs in RPL (102). Further investigation into microRNAs is warranted.

Finally, DNA methylation is another important aspect of sperm epigenetics that plays a role in male fecundity. Recent studies have reported an association between differentially methylated areas in the sperm DNA and male fecundity (103,104). Unexplained RPL as a result of early embryogenesis defect is one degree of separation away from male fecundity and it remains to be explored, the role of differential DNA methylation profiles in male partners of women with unexplained RPL.

Conclusions and future directions

RPL is a multifactorial disease and we are just beginning to scratch the surface of understanding the male contribution to unexplained RPL. The study of epigenetic biomarkers that are contributory to unexplained RPL is greatly needed. Abnormal DNA fragmentation is likely a symptom of multiple pathways; some of which we understand and some requiring significant further investigation (*Figure 3*). This is an area of research that involves an overlap between genetics, epigenetics and environmental factors. In addition, there is a growing need for more reliable tests of sperm aneuploidy and research into how to overcome this obstacle for selecting sperm for use in ART. Ultimately, the mechanisms by which these genetic and epigenetic mechanisms lead to RPL must be understood in order to develop therapeutic approaches.

Furthermore, we have only begun to explore the role of genes that have been found to be associated with male infertility in unexplained RPL. There are likely other as yet unknown genetic abnormalities unrelated to male infertility that might be implicated in unexplained RPL. Some great discoveries in science have occurred serendipitously and we cannot even begin to predict how to determine these unknown male genetic contributions. Perhaps pedigree information in couples with unexplained RPL may be helpful in identifying a subset of individuals in which the disease is strongly inherited. Linkage analysis and genome wide association studies could then ensue. This is an

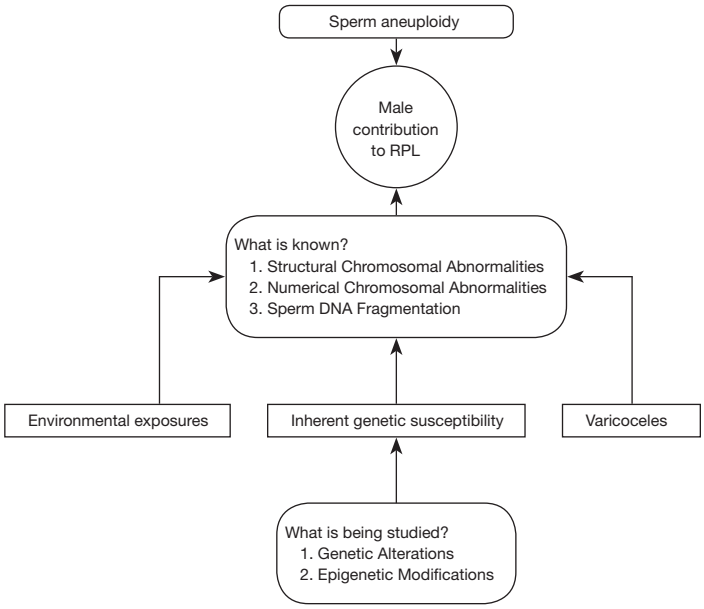

Figure 3 The male contribution to recurrent pregnancy loss.

interesting thought but would undeniably be costly and at risk of not yielding useful information.

With the information we currently have, testing for sperm chromosomal abnormalities and DNA fragmentation appears to be a reasonable option for male partners of women with unexplained RPL, with referral to genetic counselor if results are positive (69). Although, it remains difficult to predict the exact risk of unfavorable outcomes in the presence of positive findings from the available tests we have especially with the limitations in the methods of testing, this information provides for more detailed discussion about the risks and potential impacts on subsequent pregnancy outcomes. Psychologically, there may be benefit to couples in understanding the reasons for their losses, and diagnosis of a male factor may help couple in considering alternative reproductive options, including the use of donor sperm. Clinicians currently counsel couples with unexplained RPL that the chance of a livebirth in a subsequent pregnancy is approximately 75% (3). However, given the heterogeneity of unexplained RPL, this may not be accurate in a subset of patients. Further understanding of this complicated ailment will allow couples to make more educated, albeit difficult reproductive decisions.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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