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Review Article

Genetic Engineering with CRISPR-Cas9: Methodologies, Genetic Techniques, and Bioethics.

Andree-Zeid Kakish*, Bryson Martin*, Meena Adiyodi*, Connor Majewski*, G. Ian Gallicano*.

Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, 3900 Reservoir Road NW, Washington DC 20007, USA.

***Equal contributors**

Abstract

For the last ten years, the precision of genome engineering in human embryos has increased tenfold using the CRISPR-Cas9 genome editing tool. Commonly used as an intervention strategy for disease prevention, CRISPR-Cas9's precision to target disease-carrying genes far exceeds that of its predecessors, ZFNs and TALENs, and has been used for conditions like β-thalassemia, familial hypertrophic cardiomyopathy (FHC), glucose-6-phosphate dehydrogenase (G6PD) deficiency, and many others. As its utilization increases, additions to its procedure to refine its efficacy have also continued, as explained by adding homology-directed repair (HDR), high fidelity Cas9 variants, and chemical enhancers to further increase the success rate, while reducing the off-target effects. Despite current progress, the risk of genomic instability, large deletions, and variable repair mechanisms is still high. Another concern is how the current ethical and regulatory frameworks within the United States do not account for this technological advancement. This review aims to examine current CRISPR-Cas9 methodologies and genetic techniques, to evaluate the associated limitations and challenges, and to explore the ethical implications of this technique in the advancing field.

KeyWords: CRISPR-Cas9, Germline Editing, Homology-Directed Repair (HDR), Genomic Instability, Bioethics

INTRODUCTION

Over the past decade, genetic engineering has made major leaps in the development of research and in the attempt to edit the human genome. Human gene editing technologies have been at the center of ethical and clinical debates around the world, and the CRISPR-Cas9 system has revolutionized targeted modifications of DNA with precision, efficiency, and versatility. CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats) and its associated protein, Cas9 (CRISPR-associated protein 9), have changed biomedical research and become a foundation in both therapeutic and experimental applications.

Unlike earlier works of genome editing systems such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which involved engineering complex proteins for a specific target sequence, CRISPR-Cas9, discovered as part of the adaptive immune system in prokaryotes, is a system that uses a gRNA to direct the Cas9 endonuclease to a specific genomic location where it introduces a double-strand break (DSB) in the DNA. Once a DSB occurs, the endogenous repair pathway of the cell starts to repair the break either via a non-homologous end joining (NHEJ) or homology-directed repair (HDR) [1].

These methodologies have greatly advanced how genes are studied for disease modeling and therapeutic research. Further, the application of CRISPR-Cas9 has generated significant interest in human embryos as it creates new opportunities in the prevention of heritable diseases through germline modification. Researchers have been using strategies such as dual-guide RNA targeting, delivery of Cas9 as ribonucleoprotein complex, and the incorporation of synthetic donor templates for HDR [1]. Through these methodological refinements, they have reduced common limitations such as mosaicism, off-target effects, and unintended chromosomal rearrangements. Newer techniques, such as performing single-nucleotide alterations without introducing double-stranded bonds, have also been shown to minimize the risks of genomic instability, sparking optimism for potential applications. However, the extension of CRISPR technologies being used on human embryos has raised the question of profound ethical, legal, and social

***Corresponding Author:** **Andree-Zeid Kakish,** Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, 3900 Reservoir Road NW, Washington DC 20007, USA. **Email:** andreekakish@gmail.com.
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concerns. It is shown that germline modifications are heritable through future generations, thus posing the necessity for evaluating the safety and long-term consequences. Genomic editing frameworks vary across countries, showing differing societal values and how engaged the public may be. This review aims to integrate methodologies within CRISPR-Cas9 embryo editing, examine gene technological innovations, and evaluate the ethical and regulatory challenges that must be addressed before clinical translation.

METHODS AND INTEGRATION OF CRISPR-CAS9

The first study to utilize CRISPR-Cas9 in gene editing human tripronuclear (3PN) embryos was done in 2015 in an attempt to cleave an endogenous β-globin gene (HBB), a component of hemoglobin which is mutated in β-thalassemia [2]. While cleavage was successful, the efficacy of precise repair within the genome utilizing HDR was low [2]. Since then, the application of CRISPR-Cas9 has expanded to correct a variety of other disease-causing mutations in human embryonic cells. One example was in an attempt to correct a mutation in the SLC10A2 gene, which affects bile acid transport in pluripotent embryonic haploid cells [3]. After verifying the existence of the mutation and preparing for CRISPR by designing gRNA and the template, fluorescence-activated cell sorting (FACS) with Hoechst 33342 was used to isolate and culture the embryonic haploid cells [3]. Nucleofection then introduced Cas9-GFP to the cells, so that during flow cytometry, only the haploid cells that glow green, indicating successful CAS9-GFP expression, in those cells [3]. Results indicated CRISPR efficacy to be 77.1%, and 14.6% of their clones had the corrected SLC10A2 [3]. This emphasizes the high efficacy of the CRISPR-Cas9 procedure and proves how embryonic gene editing is successful for both the targeted cells and their clones post-replication.

CRISPR-Cas9 has also shown remarkable ingenuity in double-stranded breaks and homology-directed repair. One such study used CRISPR-Cas9 to correct a heterozygous mutation in the MYPBC3 gene, which is responsible for familial hypertrophic cardiomyopathy (FHC), which is a potentially fatal heart condition [4].

Researchers used donated oocytes from healthy female donors and sperm from a male that was heterozygous for the *MYPBC3* □*GAGT* mutation [4]. Fertilization of the embryo was performed using intracytoplasmic sperm injection (ICSI), which is a technique that directly injects a single sperm into the oocyte cytoplasm to ensure that editing is synchronous at the one-cell zygote stage [4]. After the ICSI was performed, Cas9 mRNA was co-injected with either a single-guide RNA (sgRNA) or a single-stranded oligodeoxynucleotide (ssODN) donor to facilitate HDR. sgRNA was designed specifically for the mutant paternal allele, along with a protospacer adjacent motif (PAM) which overlapped the mutation itself to prevent cutting of the wild-type maternal allele. This was integral in ensuring the mutated paternal allele was the only allele edited [4]. Researchers found that a majority of the embryos underwent successful correction again when using sgRNA, and no evidence was found that the ssODN repair template was used. Sequencing revealed that the wild-type maternal allele was used as a template for repair of the mutated paternal gene through interhomolog recombination [4]. The result of interhomolog repair showed high efficiency of gene correction, with up to 72% of the embryos showing correction with no mosaicism [4]. To further assess these editing outcomes, embryos were cultured to the blastocyst stage, and whole-genome sequencing and targeted deep sequencing were done to show consistency across all cells. Most embryos were found to be uniform; however, there was an indication of incomplete editing or large deletions near the target site. These off-target effects were due to alternative repair mechanisms such as microhomology-mediated end joining (MMEJ) [4]. Single-nucleotide polymorphism (SNP) phasing was also done to assess the maternal origin of repair sequences for further verification of true interhomolog recombination. No evidence of exogenous template incorporation in those embryos was found, which further solidified the idea that the primary repair template was through maternal DNA [4]. These findings suggest that when using CRISPR, early human embryos may favor interhomolog HDR, especially after direct fertilization. Although this study showed significant improvements in DNA repair through CRISPR, there was some caution for clinical application, such as challenges and understanding how some embryos underwent abnormal repair and what long-term safety of these edits looks like. This study provided a framework for CRISPR in the potential of safe germline correction of certain monogenic diseases [4]

Further evidence shows detailed exploration of how the choice of Cas9 variants and repair pathways influences their efficacy and efficiency of genome editing and the safety of human embryos [5]. While wild-type Streptococcus pyogenes (SpCas9) is the most common endonuclease used, one research article reviews the highlights and growing interest in high-fidelity variants that were engineered, such as SpCas9-HF1 and HypaCas9 [5]. These two variants have an amino acid substitution in Cas9's REC3 and RuvC domains, which essentially weaken nonspecific interactions between Cas9 and DNA. This would improve cleavage precision without ultimately reducing on-target activity [5]. In the embryo editing experiments, SpCas9-HF1 had a sharp decline in off-target activity, which was quantified using CIRCLE-seq and SITE-seq, which are genome-wide unbiased tools. SpCas9-HF1 maintained high efficacy at loci such as HBB and EMX1 [5].

Another significant point is how to overcome the bias between the two dominant DNA repair pathways that are activated following Cas9-induced DSB: HDR and NHEJ [5]. While HDR

is used for precise gene correction when codelivered with ssODNS, its activity has been shown to be suppressed in the early embryonic stages due to low endogenous RAD51-ssDNA. The research overcame this obstacle by using a chemical adjuvant such as RS-1, which stabilized the RAD51-ssDNA filaments to favor HDR and Scr7, a DNA ligase IV inhibitor that suppresses the action of NHEJ [5]. Through this study, although interembryo variability remained high, embryos treated with RS-1 showed a statistical increase in HDR efficiency (approximately 15% more efficient) when editing loci such as MYBPC3 and HBB [5]. These compounds were used with caution as they were susceptible to developing arrest or cytotoxicity if the dosing was not optimal [5].

METHODS AND INTEGRATION OF NEW STUDIES

Additional investigations have expanded on foundational CRISPR techniques by demonstrating successful gene correction in viable 2PN human zygotes. For example, Tang et al. injected Cas9 protein complexed with sgRNAs and HDR templates into single-cell embryos, targeting pathogenic mutations in the HBB and the glucose-6-phosphate dehydrogenase (G6PD) genes [6]. Their results provided proof-of-concept that CRISPR-Cas9 could be applied effectively to normal human embryos, with successful gene correction achieved through HDR. Although the technique showed promise, the authors acknowledged correction-specific limitations and emphasized the need for further research to optimize repair fidelity and safety [6].

Other work investigating how CRISPR-induced DSBs are repaired in embryos reported that these breaks introduced into heterozygous loci in preimplantation embryos were often repaired via gene conversion using the homologous wild-type allele [7]. While this can restore a functional genotype, these conversion tracts often span well beyond the intended site and lead to unexpected loss of heterozygosity (LOH). This highlights that although embryos are capable of borrowing sequence from a healthy allele to accurately repair a mutation, gene conversion events may unpredictably extend beyond the target site, leading to unintended genomic alterations.

Further mechanistic insight comes from studies assessing allele-specific editing. Zuccaro et al. targeted a frameshift mutation in the paternal allele of the EYS gene and found that although some embryos were corrected via MMEJ, approximately half of the induced DSBs went unrepaired, resulting in loss of chromosomal arms or hemizygous deletions due to off-target cleavage [8]. These results reveal the variable nature of DNA repair outcomes, even when targeting a single allele in otherwise viable zygotes.

These studies build on prior foundational work and demonstrate the potential for precise gene correction and technical challenges that complicate CRISPR-based editing in early human embryos. Repair outcomes remain highly sensitive to variables including zygotic stage, allele context, and double-strand break location.

CRISPR-Cas9 has also been applied to investigate the regulatory roles of non-coding RNA's during development. In a recent study, CRISPR was used by researchers to oblate individual microRNAs (miRNA) within the same family, revealing that these miRNAs despite sharing similar sequences had different effects on cardiac differentiation [9]. This approach showed that CRISPR can be used to precisely interrogate post-transcriptional gene regulation while also correcting protein coding genes. By targeting individual miRNAs, the study showed that specific non-coding elements played distinct rules in direct lineage commitment [9]. These findings show that CRISPR's utility in developmental biology serves as an important understanding in gene regulatory networks at a fine scale. Together, the current research emphasizes optimizing template design, minimizing mosaicism, and improving detection of unintended alterations before the mentioned methods can be considered for clinical use. A selection of studies cited in this section is summarized below to highlight differences in gene targets, editing strategies, and observed outcomes (**Table 1**).

Table 1. Summary of representative CRISPR-Cas9 studies in human embryos or stem cell models cited in this review, including their target genes, cell types, editing outcomes, and relevant references.

Target Gene	Condition Modeled or Treated	Cell Type / Model	Repair Outcome	Reference
HBB	β-thalassemia	3PN human zygotes	Low HDR, mosaicism, off-target effects	[2], [6]
SLC10A2	Bile acid transport defect	Haploid hPSCs	77.1% Cas9 efficacy, 14.6% HDR-corrected clones	[3]
MYBPC3	Familial hypertrophic cardiomyopathy (FHC)	Human embryos (ICSI fertilized)	Interhomolog HDR, 72% correction, no mosaicism	[4]
EMX1	n/a (off-target analysis)	Human embryos	High-fidelity Cas9 shows reduced off-target cuts	[5]
EYS	Retinal degeneration (frameshift mutation)	Human embryos	MMEJ, 50% unrepaired DSBs, chromosomal loss	[8]

POU5F1 (OCT4)	Pluripotency factor	Human embryos	LOH, segmental chromosomal abnormalities	[10]
PLCZ1	Infertility (sperm activation failure)	Human embryos	Successful HDR correction, low LOH, reduced mosaicism	[15]
miRNA family	Cardiac differentiation	Stem cells	Discrete lineage-specific regulation uncovered	[9]

SCIENTIFIC AND TECHNICAL LIMITATIONS

Despite early optimism, a growing body of research has revealed significant risks associated with CRISPR-Cas9 editing in human embryos. One major concern is unintended genomic instability, including large deletions, LOH, and chromosomal aberrations at or near the target site. Alanis-Lobato et al. used single-cell genomics and transcriptomics to study human embryos edited at the POU5F1 (OCT4) locus and found that approximately 16% of edited cells exhibited segmental chromosomal abnormalities or LOH spanning 4-20 kb [10]. Many of these outcomes were undetectable by conventional polymerase chain reaction (PCR), suggesting that current validation methods underestimate the true frequency of unintended effects.

Zuccaro et al. further documented extensive LOH and unrepaired DSBs in embryos edited at heterozygous loci [8]. They showed that Cas9-induced breaks on a paternal allele could result in loss of one or both chromosomal arms in nearly half of the embryos examined. Similarly, the study found that gene conversion and NHEJ were dominant repair mechanisms, sometimes leading to identical indels on both alleles or unpredictable large-scale genomic alterations.

Even in cases where editing appears successful, follow-up genomic analyses often reveal unintended outcomes, including mosaicism, large deletions, or ambiguous repair signatures. This is evident in the widely cited study by Ma et al. (2017), which concluded that interhomolog HDR from the maternal allele efficiently corrected a pathogenic mutation in MYPBC3. Despite these findings, the study's reliance on bulk sequencing limits its ability to fully determine whether the repair events resulted from homology-directed repair or gene conversion, and may obscure low-level mosaicism or allelic diversity. Without rigorous long-read sequencing or single-cell analysis, important repair signatures may be inaccurately characterized or overlooked.

The cumulative evidence suggests that while human embryos can repair DSBs through endogenous pathways, the outcomes are highly variable and frequently involve unintended alterations. Repair mechanisms such as MMEJ, NHEJ, and gene conversion lack the precision required for clinical translation, especially when editing must be confined to the single-cell stage to avoid mosaicism. Furthermore, the inability to fully assess all cells in an embryo before implantation presents a serious ethical and safety concern.

In summary, these findings highlight that CRISPR editing in human embryos remains limited by both methodological and biological constraints. Addressing these challenges will require enhanced delivery techniques, improved repair pathway modulation, and comprehensive post-editing validation across all embryonic cells before any clinical use can be responsibly considered.

Ethical Implications

Prior to 2015, all therapeutic applications of genome editing were conducted in somatic cells, until Huang and his team published their research, which raised new bioethical concerns pertaining to the modification of the human germline [2]. Since then, CRISPR-Cas9, although becoming widely studied, has fragmented legal and regulatory landscapes surrounding heritable genome editing [11]. Such legal and regulatory implications have been assessed using detailed case studies and global comparisons. One such study has classified the governance of using CRISPR-Cas9 into four categories: permissive (China pre-2018), restrictive (most EU countries), Intermediate with tight regulation (UK), and undeclared (developing nations) [11]. A major turning point that exposed gaps in regulatory enforcement of using CRISPR-Cas9 was highlighted in the 2018 He Jiankui incident. In this incident, CRISPR was used to genetically modify babies in China [11].

This prompted China to revise its biosecurity law and establish review systems that focused on ethics, which required government registration of all gene-editing projects [11].

In the UK's approach, they say progressive oversight and using a type model, with the Human Fertilisation and Embryology Authority (HFEA) requiring licensing in a comprehensive ethical review that is strict when researching on human embryos. This restriction is up to 14 days post-fertilization [11].

In stark contrast, the United States authorizes a de facto ban on germline editing in a clinical setting that has restrictions placed on funding from the Food and Drug Administration (FDA) and National Institutes of Health (NIH), even if no legal prohibitions are found in their work [11]. Within the same research, United Nations Educational, Scientific and Cultural Organization's (UNESCO) call for a global moratorium and the World Health Organization's (WHO) 2021 Recommendation push for a registry in the ethics oversight community that is international [11]. However, there have been debates on whether this international consensus would cause cross-border exploitation. This research states that multilateral governance systems supported by transparent public discourse and regulation that adapts to responses would ultimately aid in understanding the ethics of CRISPR-Cas9 [11].

Risk of Harm and Potential Benefits

While CRISPR-Cas9 and earlier genome editing techniques like ZFNs and TALENs have emerged as transformative tools that have expanded our ability to study and manipulate model organisms, they are not without risk. According to an early study by Gaj et al. ZFNs and TALENs pioneered the ability to edit genes at precise locations through DNA cleavage, but concerns for specificity, off-target effects, cytotoxicity, and large sizes of sequences presented challenges to widespread adoption [12]. Within the same study, researchers found that CRISPR-Cas9, with its RNA-guided mechanism, could overcome many of these challenges by offering a more concise delivery method for gene editing (Gaj et al., 2013). Despite these improvements, the researchers advocated for further evaluation of the specificity and toxicity of RNA-guided DNA endonucleases in vitro and in vivo, as well as continued study of off-target effects in order to better understand potential risks of CRISPR-Cas9.

Notably, Liang et al. (2015) demonstrated that CRISPR-Cas9 could introduce intended genetic modifications in 3PN zygotes. However, the researchers reported significant mosaicism and off-target cleavage, emphasizing that while gene correction was achievable, unintended consequences were frequent and unpredictable [2]. Furthermore, the whole-exome sequencing only covered a small portion of the genome and most likely underestimated the off-target effects in 3PN zygotes [2]. Another study found that the CRISPR-Cas9 system has significant off-target effects that can induce a wide range of indels, with a large number of one-base insertions and a few large deletions, which can cause a significant potential of mutagenesis and chromosomal rearrangements [13]. These findings underscore a central risk when it comes to gene editing: that embryos edited using CRISPR may carry undetected genomic alterations that could affect not just one individual, but future generations as well.

Recent work by Liang et al. provided additional evidence for these concerns [14]. Their comprehensive single-cell analyses revealed frequent large deletions, gene conversions, and LOH after editing attempts, even when employing high-fidelity Cas9 variants. They go on to discuss that gene conversion outcomes most likely go unnoticed in most gene editing studies, since this can only be proven by the detection of LOH at flanking heterozygous loci. Mosaicism often masks gene conversion in pooled DNA samples, though, so single-cell analysis is required (Liang et al., 2023). These unexpected and unpredictable mutations complicate the potential benefits of genome editing, raising concerns about the long-term health effects on edited individuals. Without robust methods to predict or eliminate such unintended effects, the use of CRISPR in embryos remains ethically and scientifically precarious, requiring further investigation of CRISPR-Cas9 gene editing before clinical applications.

Nevertheless, some studies have demonstrated the potential for positive outcomes. Bekaert et al. showed successful correction of an infertility-related point mutation in PLCZ1 in human embryos without any observed loss of the targeted chromosome, excluding the hypothesis that DSB induction in human embryos is responsible for partial chromosome loss [15]. However, observance of short-range LOH events and mosaicism, while reduced, could result in detrimental outcomes such as expression of recessive alleles, which has been seen in cancer and epigenetic imprinting disorders (Bekaert et al., 2023). While the enhanced delivery method and improved guide RNA design allowed for correction of a heterozygous basepair substitution in PLCZ1, the occurrence of complex genetic outcomes, such as LOH and mosaicism, shows that there is still much to learn about CRISPR-Cas9.

Such outcomes of CRISPR-Cas9 gene editing highlight the delicate balance between profound risk and unprecedented medical advancements. Until technical refinements can fully ensure safety and reliability, and until such mechanisms exist to carefully monitor edited embryos across their development, clinical application remains premature.

CONCLUSION: FUTURE DIRECTIONS AND BALANCING INNOVATION WITH CAUTION

Looking forward, the future of embryo editing with CRISPR-Cas9 must prioritize both technical optimization and ethical obligations. Studies by Gaj et al. and Liang et al. suggest that technical challenges such as off-target mutations, large deletions, and mosaicism, while formidable, are not insurmountable [12][14]. Newer innovations, such as base editing and prime editing, offer more precise alternatives to traditional DSB-dependent CRISPR-Cas9 approaches, potentially minimizing unintended consequences of gene editing in edited embryos and future generations (Liang et al. 2023).

For instance, work on positive mutation correction strategies demonstrates how thoughtful design of repair templates, careful timing of CRISPR delivery, and use of chemical enhancers can improve HDR outcomes (Liang et al., 2023). In addition, they advocate for single-cell sequencing and whole-genome validation protocols to become standard practice to detect rare or subtle genomic alterations before the edited embryos are considered for implantation (Liang et al., 2023)

Beyond technical limitations, however, there is still much debate on the ethics of gene editing in human embryos that requires a broader societal conversation. While such technology promises to expand our ability to explore and alter any genome in order to understand and treat genetic diseases, we must take caution against propagating eugenics. One systematic review compared 223 different publications and found that 26.9% of them addressed eugenics as

an ethical consideration, and the two major themes that emerged focused on enhancement and disability [16]. While suggestions to remove the presence of disability were proposed and greatly supported, many authors advocated for the inclusion of individuals with disabilities and disability rights groups to find balance in the editing [16]. On the other hand, discussion of enhancements and how it applies to cognitive, physical, and behavioural changes was met with more reluctance [16]. Many view such enhancements as a misuse of genome editing, and fear that it will result in society leaning into an eugenic mindset and away from embracing the existing genetic diversity.

Therefore, when considering the applications of CRISPR-Cas9 in human embryonic gene editing, coupling it with HDR, high-fidelity Cas9 variants, and chemical enhancers serves to increase both the precision and efficacy of the tool. Moving forward, this procedure must be further developed to overcome the technical shortcomings that currently exist with the procedure, including genomic instability, large deletions, and variable repair mechanisms. Additionally, finding ways to enhance delivery techniques and repair pathway modulation in the procedure is also greatly beneficial. The use of CRISPR-Cas9 in human embryos is still a highly controversial topic due to the fear that its increased usage will encourage eugenics in an immoral manner, one that relies more on enhancement rather than on disease prevention. This makes establishing regulations on both the research and utilization of CRISPR-Cas9 all the more important to ensure all ethical guidelines are being met, and finding ways to standardize that globally will be effective in the coming years.

Beyond embryo editing, CRISPR-Cas9 also shows unique potential when combined with non-embryonic stem cell therapies such as clinical treatments of Type 1 diabetes. When induced pluripotent stem cells (iPSCs) were derived from adult tissues, insulin producing β cells were generated by researchers that showed fewer ethical concerns and reduced tumorigenic risks compared to embryonic stem cell generations [17]. CRISPR has allowed for precise therapies without relying on viral vectors and enabling target corrections of disease causing mutations [17]. iPSC-derived β cells have successfully restored insulin function within animal models which in theory demonstrates the translational promise of this approach [17]. These advances highlight how CRISPR based therapies can be applied ethically and effectively in regenerative medicine. In a related application, Albitar et al. demonstrated how CRISPR-Cas9 can be harnessed to model complex cancer genomes in human stem cells through multiplexed editing and HDR-based correction of driver mutations such as ASXL1 [18]. Their work highlights the ability of CRISPR to enable the study of tumor progression and therapeutic gene restoration without crossing the ethical boundary of germline editing. These findings illuminate the value of CRISPR not only as a therapeutic tool, but as a platform for disease modeling and precision oncology. In conclusion, while CRISPR-Cas9 holds great potential, its clinical application in human embryos must be guided by rigorous science and ethical oversight.

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Author information

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Ethics declarations

Competing Interests

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