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CRISPR-Cas12: A Versatile Tool for Genome Editing and Beyond

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ABSTRACT

CRISPR-Cas systems are emerging gene editing toolkits that have been used for the manipulation of DNA and RNA, among which CRISPR-Cas12 demonstrates superior properties in terms of target DNA engineering *in vivo* compared to CRISPR-Cas9 as well as nucleic acid detection compared to CRISPR-Cas13. A comprehensive understanding of the mechanisms and emerging applications of CRISPR-Cas12-based technologies is critical for maximizing their benefits across various fields, including basic science, medicine, and food safety. In this study, we review the molecular features of both natural and engineered CRISPR-Cas12 compared to CRISPR-Cas9 and CRISPR-Cas13, and we provide an overview of established CRISPR-Cas12-based genome editing and diagnostic platforms. Furthermore, we discuss the advantages, challenges, and prospects for future directions of CRISPR-Cas12 in gene editing and nucleic acid detection.

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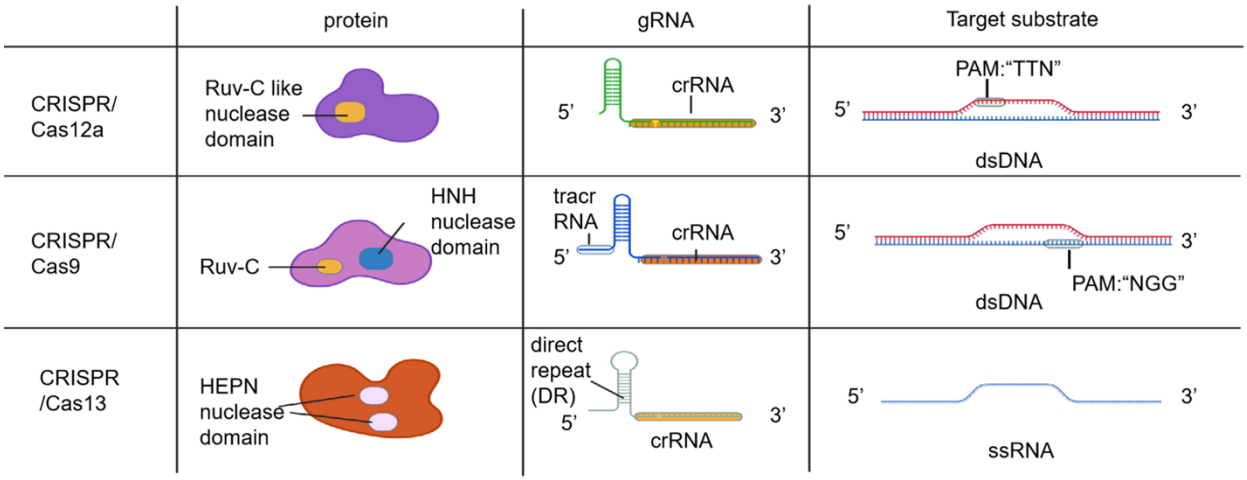
Introduction

The purpose of gene editing is to alter specific gene expression or manipulate the sequences of targeted genes by deleting, inserting, or replacing desired bases within the DNA sequence. Currently, a significant number of gene editing platforms have been developed, particularly those based on the "zinc finger nuclease" (ZFNs) developed by Chandrasegaran *et al.* in 1999 [1], transcription activator-like effector nucleases (TALENs) first reported by Boch *et al.* in 2009 [2], as well as Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system, identified in 1987 by Ishino *et al.* [3] and engineered by Jinek *et al.* for gene editing in 2012 [4], to achieve an efficient and accurate gene editing. For instance, ZFNs combine engineered zinc finger DNA with non-specific ribozymes, which can create DNA double-strand breaks (DSBs) at the desired DNA sites, thereby activating the DNA repair system to treat disease [5]. In addition, TAL (transcription activator-like) effectors identified from the plant pathogenic Xanthomonas can specifically bind to DNA, and TAL-based nucleases can accurately cleave the target DNA sequence following recognition [2, 6]. Although the aforementioned gene-editing tools have successfully achieved targeted genome modification, there remains a need for simpler, more efficient, and more specific technologies. The CRISPR-Cas systems represent more convenient and effective gene-editing tools. They use CRISPR-associated proteins (Cas) to change the DNA base sequence under the guidance of guide RNA [7]. CRISPR-Cas systems are classified into two main classes: Class 1 and Class 2, which are further subdivided into six categories, ranging from Type I to Type VI [8]. The key distinction between Class 1 and Class 2 lies in the number of effector Cas proteins required for gene editing. Class 1, which includes Types II, V, and VI, necessitates multiple effector proteins during the gene-editing process. In contrast, Class 2, which encompasses Types I, III, and IV, typically consists of a single effector protein in its effector complexes.

CRISPR-Cas12 system belongs to Class 2 CRISPR-Cas systems that consist of CRISPR-Cas9, CRISPR-Cas12, and CRISPR-Cas13 systems [9]. It consists of a guide RNA that recognizes the specific single-stranded DNA (ssDNA) and the Cas12 pro-

Figure 1. Structural differences among CRISPR-Cas12, CRISPR-Cas9, and CRISPR-Cas13. The distinctions among these CRISPR-Cas systems include the domains of Cas proteins, gRNAs, recognized substrates, and collateral activity. Specifically, the Cas12 protein contains a RuvC domain, Cas9 possesses both a RuvC domain and an HNH domain, while Cas13 proteins feature an HEPN domain. The recognition mechanism of CRISPR-Cas9 requires a tracrRNA. The substrates of CRISPR-Cas12 are ds/ssDNA with "TTN" PAM, CRISPR-Cas9 recognizes dsDNA with "NGG" PAM, while CRISPR-Cas13 targets ssRNA and no PAM is required. Both CRISPR-Cas13 and CRISPR-Cas12 exhibit collateral activity, whereas CRISPR-Cas9 does not. The figure was created using BioGDP (BioGDP.com).



tein, which is responsible for targeted DNA cleavage [10] (**Figure 1**). Different from CRISPR-Cas9, which specifically recognizes and cleaves double-stranded DNA (dsDNA) without collateral effect [4], and CRISPR-Cas13, which targets RNA [11], the specificity of CRISPR-Cas12 in recognizing dsDNA and ssDNA, along with its unique characteristics, makes it a powerful tool for ssDNA editing and detection. This is particularly important for the elimination and detection of viruses, which pose a significant threat to human health. Rapid, accurate, and specific detection of viruses lurking in the human body is crucial for preventing and controlling the emergence and spread of infectious diseases, such as Coronavirus disease (COVID-19) [11-13]. Infectious pathogens can be efficiently and specifically detected by identifying their nucleic acids [14]. Current nucleic acid detection methods include PCR technology, CRISPR technology, and others. The CRISPR-Cas system has garnered significant attention due to its immense potential in nucleic acid detection, as well as the convenience and ease of use of its existing detection platforms [11]. In this article, we review the development of the CRISPR-Cas12 system and highlight its unique features in the latest applications for gene editing and nucleic acid detection.

1. Characters of CRISPR-Cas12 system

1.1 The development and original role of CRISPR-Cas system

The CRISPR array was first reported in 1987 [3] , and the CRISPR-Cas gene was discovered in 2002 [15], which is derived from the immune systems of bacteria and archaea that defend against RNA viruses [16]. The immune components of the CRISPR system include CRISPR-effector proteins and CRISPR RNA (crRNA). The immune process of the CRISPR-Cas system is divided into three steps. *Adaptation*: The Cas protein recognizes and intercepts the base fragment of the target DNA, subsequently inserting it into the CRISPR array. *Processing*: Matured crRNA is formed through the expression and processing of precursor RNA (pre-crRNA). crRNA then assembles with corresponding Cas proteins to form an RNA-guided endonuclease complex. *Interference*: During the interference stage, once the matured crRNA recognizes the Protospacer Adjacent Motif (PAM) that corresponds to the target sequence, it binds to the target DNA, prompting the Cas effector protein to initiate its endonuclease function [17]. Twenty-five years after the initial description of CRISPR, the CRISPR-Cas9 system was adapted for gene editing in 2012, marking the beginning of a new

era in gene editing with CRISPR-Cas systems [18-20]. Notably, CRISPR-Cas12, which belongs to Class 2, Type V CRISPR systems and was originally named Cpf1 (derived from the CRISPR system of Prevotella and Francisella), has gained attention for its superior capabilities in DNA gene editing and nucleic acid detection [21].

1.2 Differences of CRISPR-Cas12 with CRISPR-Cas9

Although CRISPR-Cas12a and CRISPR-Cas9 both belong to Class 2 of the CRISPR system and share many similar functions and protein sizes, there are significant differences in their crRNA, recognition sequences, and cutting mechanisms. This article will compare these differences in detail. Firstly, while CRISPR-Cas9 recognition requires both trans-activating crRNA (tracrRNA) and crRNA to form a ribonucleoprotein (RNP) complex for target recognition and endonuclease activity, CRISPR-Cas12a only requires a mature crRNA to perform its function. The Cas12a protein exhibits both ribozyme and endonuclease [4]. After the long pre-crRNA binds to its ribonuclease site, the Cas12a protein processes the pre-crRNA into mature crRNA. Therefore, in this regard, CRISPR-Cas12a has a more streamlined composition. Secondly, The PAM recognized by CRISPR-Cas9 is usually located downstream of the target DNA spacer sequence and on a non-template strand, usually recognizing the sequence 5'-NGG-3'. In contrast, the PAM for CRISPR-Cas12a is situated upstream of the template strand interval sequence of the target DNA. This PAM is characterized by a thymine (T)-rich sequence, typically 5'-TTN/TTTN/TTTV-3'. Upon recognizing the PAM, the CRISPR-Cas12a complex can initiate cleavage of the target sequence. Thirdly, the Cas12a protein functions as a single ribozyme site capable of cleaving dsDNA at the same location, resulting in interlaced DSBs. This staggered DSB configuration offers a distinct advantage in the subsequent DNA repair process. In contrast, CRISPR-Cas9 typically generates a blunt DSB. Fourthly, there are notable differences in the protein structures of the two systems. Both Cas12a and Cas9 possess a characteristic RUV-C-like nuclease domain; however, Cas9 also includes an HNH (His-Asn-His endonuclease) domain, while Cas12a features a simpler RuvC (homology domain of UV-sensitive gene product C activity for resolving Holliday junction) domain [22]. Additionally, the activated Cas12 protein can bind to substrate DNA and cleave arbitrary free single-stranded DNA (ssDNA), a property referred to as collateral cleavage, which is essential for nucleic acid detection [23]

1.3 Differences of CRISPR-Cas12 with CRISPR-Cas13

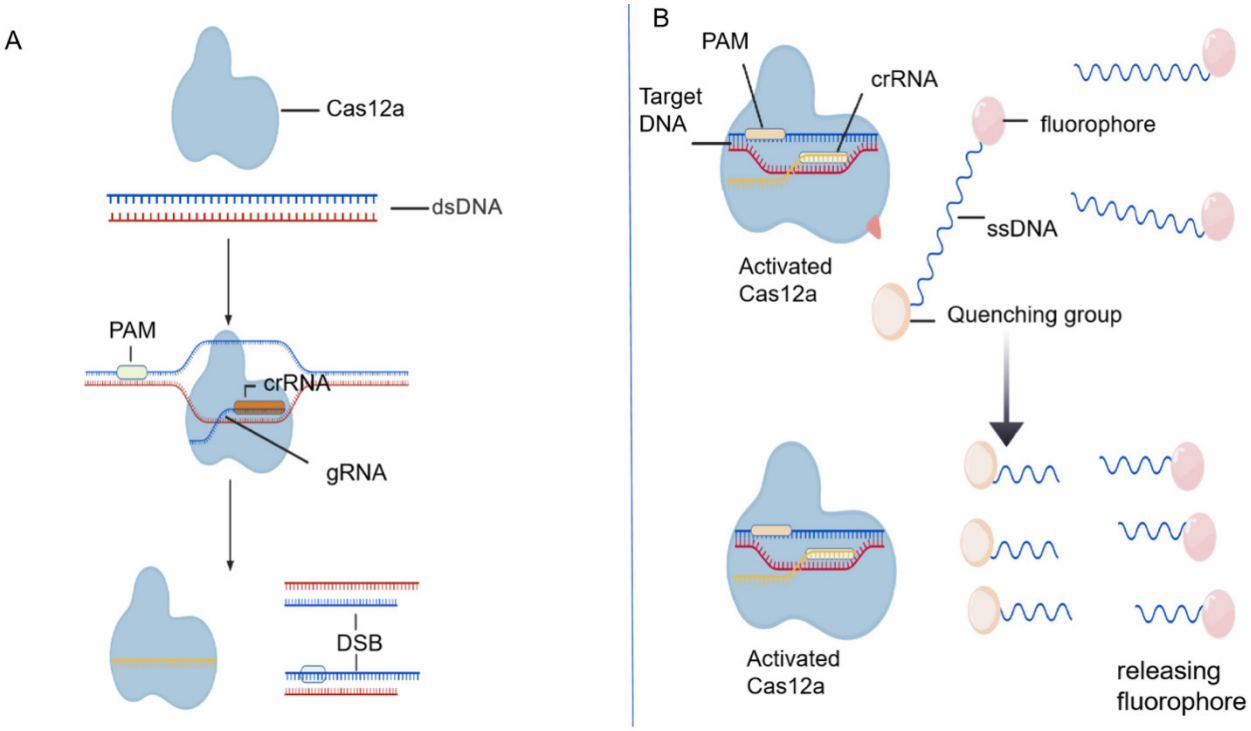
In addition to the DNA-targeting CRISPR-Cas systems, the CRISPR-Cas13 system is a more recently developed and widely used RNA-targeting technology [24], which is extensively employed for RNA targeting and detection [24, 25]. Both CRISPR-Cas13 and CRISPR-Cas12 exhibit active side-cutting activity and can cleave single-stranded nucleic acids after binding to substrate DNA or RNA. Consequently, both systems have been engineered for various nucleic acid detection applications based on this property [26]. However, there are significant differences between these two CRISPR-Cas systems. The substrates recognized and cleaved by Cas12a are dsDNA and ssDNA, making it a DNA-targeted ribozyme system. In contrast, the substrate for the Cas13 protein is ssRNA, which is usually used for cutting viral RNA and plays an immune defense role against foreign RNA in the cytoplasm. Because of their differing substrates, these systems also possess distinct structural domains. Cas13 contains two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains that facilitate RNA cleavage upon target recognition [27], while the Cas12 protein features a single RUV-C-like domain [22]. Additionally, CRISPR-Cas12a is capable of unwinding double-stranded DNA, which allows the crRNA to bind to internal target sequences containing PAM, while CRISPR-Cas13 can only recognize and cleave RNA regions with accessible structures.

2. CRISPR-Cas12 in gene editing

2.1 Application of CRISPR-Cas12 for gene editing

CRISPR-Cas12a functions as a gene editing tool by identifying and cleaving the target DNA sequence, resulting in interlaced DSBs (**Figure 2A**). This process activates the DNA repair mechanisms within the target sequence to facilitate gene editing, serving a role similar to that of CRISPR-Cas9 to some extent. The primary mechanisms of cellular DNA repair are homology-directed repair (HDR) and non-homologous end joining (NHEJ) [28]. Compared to NHEJ, HDR can utilize template DNA at the break site or unbroken homologous sister chromatids to repair DSBs. Consequently, HDR has a lower probability of

Figure 2. Mechanisms of gene editing and nucleic acid detection that underpin the applications of CRISPR-Cas12. (A) Canonical CRISPR-Cas12a recognizes and cleaves target DNA with the guidance of gRNA, thereby activating DSB repair, which facilitates DNA frameshift editing through insertion and deletion. (B) The collateral activity of the Cas12 protein requires the presence of targeted substrate DNA. This collateral effect is initiated after gRNA recognizes and binds to target sequences, resulting in the cleavage of reporter ssDNA (e.g., ssDNA reporters tagged at the N-terminus and C-terminus with 6-FAM and BHQ-1 quencher, respectively), which subsequently releases fluorescence signals. The outcomes of collateral cleavage can be assessed through fluorescence measurement. The figure was generated with BioGDP (BioGDP.com).



error and can more effectively introduce the desired gene sequence into the target DNA. The interlaced DSBs produced by CRISPR-Cas12 are frequently repaired via HDR, which enhances CRISPR-Cas12a's potential as a gene editing tool with greater accuracy than CRISPR-Cas9. Based on the aforementioned advantages, significant advancements in gene editing utilizing CRISPR-Cas12 have been made. Researchers have successfully implemented genome editing and optimized crRNA scaffolds in both human cells and animal models [29-32]. In addition to its applications *in vitro* and animal models, the CRISPR-Cas12 system also holds considerable promise for plant gene editing. For instance, studies comparing CRISPR-Cas12 with other gene-editing techniques in rice and Arabidopsis have demonstrated that the CRISPR-Cas12 system possesses substantial potential for plant genetic engineering [33]. Furthermore, researchers from the Institute of Molecular Biosciences at Mahidol University in Thailand are exploring the use of the CRISPR-Cas12 system to achieve more precise gene editing in the pinhole Aspergillus strain TBRC 277, an industry-relevant cell factory. This approach could enable the modification of industrial strains to produce higher-value biologics [34].

2.2 Challenges of CRISPR-Cas12 for gene editing in clinics

Although CRISPR-Cas12 has numerous gene-editing applications, several challenges must be addressed for its clinical applications. Firstly, off-target effects are prevalent within the CRISPR family [35], despite the Cas12 protein family exhibiting a lower likelihood of off-target activity compared to Cas9. Consequently, when CRISPR-Cas12 is utilized in human genetic engineering, there is a risk of mutations occurring in sequences other than the intended target, which significantly heightens the potential for unknown gene mutations. Recent studies are focused on developing optimal tools to predict all low-probability off-target events. For instance, the AID-SeQ-based high-throughput pool miss detection strategy,

combined with pooled-seq's large amount of missing data to create an accurate prediction model for CRISPR-Cas9 [36]. Employing similar methodologies to construct off-target prediction models for CRISPR-Cas12 offers a promising avenue for addressing this issue in the future. The second challenge in CRISPR-Cas system delivery is the difficulty associated with the large size of Cas nucleases, which poses a significant obstacle to CRISPR-based gene editing. The Cas12 protein family is structurally simpler than other Cas protein families, presenting an opportunity for innovation. Future efforts should focus on utilizing these compact Cas12 proteins to develop effective delivery vehicles, in addition to traditional methods such as viral vectors and physical electroporation. Potential strategies include the direct delivery of Cas proteins, the use of extracellular vesicle (EV) technology, and the development of intelligent nanoparticle technology [37-39]. Additionally, ethical reviews are a significant concern in the field of human gene editing. Currently, addressing the ethical issues associated with gene editing presents a substantial challenge [40, 41]. When CRISPR-Cas12 is applied to gene editing, ethical risks emerge as a critical issue that researchers must consider. The potential risks, unintended consequences, and problems arising from misuse necessitate continuous discussion and scrutiny from ethics experts and researchers.

3. CRISPR-Cas12 in nucleic acid detection

3.1 Application of CRISPR-Cas12 for nucleic acid detection

The presence of nucleic acids serves as a marker for numerous diseases, particularly infectious diseases [42, 43]. The rapid, cost-effective, and easily accessible detection of these nucleic acids, which does not require highly specialized personnel, undoubtedly benefits all of humanity. In recent years, researchers have developed several nucleic acid detection tools, among which the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) platform, based on CRISPR-Cas13, has demonstrated promising potential for RNA detection, such as COVID-19 [44]. Researchers have also recognized the Cas12a protein, which exhibits a similar collateral cleavage effect to Cas13, leading to an increasing number of applications for CRISPR-Cas12a in nucleic acid (Figure 2B). For instance, in 2018, Jennifer A. Doudna and her team co-developed the DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) [10]. This platform primarily utilizes fluorescence reporter molecules. Upon identifying and binding to the target sequence, the Cas12 protein cleaves the single-stranded DNA (ssDNA) reporter molecule linked to the fluorophore, thereby releasing the fluorophore. The concentration of nucleic acid molecules can then be determined by measuring the fluorescence intensity. This method has demonstrated the ability to rapidly and accurately identify human papillomavirus in patient samples, offering a novel strategy for simple, highly sensitive, and specific nucleic acid detection. Additionally, platforms such as HOLMES (One-HOur Low-cost Multiplexed detection platform), and Split Activator for Highly Accessible RNA Analysis (SAHARA), among others, have been developed based on CRISPR-Cas12a [45, 46]. These nucleic acid detection platforms have been introduced in succession and have quickly found applications in the detection of infectious diseases, particularly viral infections [47]. Beyond pathogen detection, CRISPR-Cas12-based diagnostic tools are also employed to diagnose and monitor other diseases, such as cancers, by assessing the levels of cancer biomarkers, including microRNAs [48-51]. Notably, the potential applications of CRISPR-Cas12-based detection platforms extend beyond medical conditions, with promising implications in fields such as food safety, thereby contributing to the advancement of CRISPR-Cas12-based toolkits [52].

3.2 Perspective of CRISPR-Cas12 for nucleic acid detection

Although the nucleic acid detection method developed based on the CRISPR-Cas12 system offers numerous advantages, several challenges impede its broader application. As previously mentioned, the recognition of the desired sequence by CRISPR-Cas12 necessitates the presence of a PAM within the targeted DNA sequence. While Cas proteins permit a certain degree of mismatch, this tolerance primarily depends on the number of mismatches and their positions relative to the PAM. Consequently, the range of target sequences that can be identified remains limited. Engineering the CRISPR-Cas12 system to recognize a wider variety of PAM sequences while preserving specificity is an urgent challenge that needs to be addressed. Indeed, a PAM-free CRISPR-Cas12 system has been proposed [53, 54], but it requires further experimental evidence to vali-

date its specificity and efficiency *in vivo*. Additionally, accurate PAM recognition modeling is also helpful in helping CRISPR-Cas12's requirements for PAM in subsequent applications [55]. Lastly, most existing nucleic acid detection platforms based on CRISPR-Cas12 rely on fluorescence signals to indicate the nucleic acid content in a sample. While this method provides an intuitive comparison of content, it poses challenges for absolute quantification analysis, despite several studies proposing potential solutions for quantification [56, 57].

Discussion

CRISPR-Cas12, a prominent protein within the current CRISPR-Cas systems, offers several advantages that distinguish it from other congeners. Firstly, CRISPR-Cas12 requires only a single crRNA for its function, eliminating the need for tracrRNA mediation. This simplification means that only crRNA factors must be considered when designing gene editing and nucleic acid detection toolkits, significantly reducing the engineering complexity and making CRISPR-Cas12 more versatile than CRISPR-Cas9, as with accumulating schemes for designing crRNA being proposed [58-60]. Secondly, as mentioned above, the PAM region recognized by CRISPR-Cas12 is a T-rich region, specifically "TTN/TTTN/TTTV" (N = A/T/C/G; V = A/C/G) [17], which is more flexible than the "NGG" recognized by CRISPR-Cas9, allowing CRISPR-Cas12 to access regions of the genome that are difficult for CRISPR-Cas9. Besides, CRISPR-Cas12 assembles a complex with designed crRNA that is easily loaded by existing transport vehicles due to its smaller size compared to CRISPR-Cas9, particularly in viral delivery systems with limited packaging capacities. Furthermore, CRISPR-Cas12 demonstrates a lower off-target potential, which minimizes the risk of unintended cuts in undesired sequences during applications, resulting in a higher fault tolerance rate [61]. Additionally, while both CRISPR-Cas12 and CRISPR-Cas13 exhibit similar collateral effects, making them ideal toolkits for nucleic acid detection, CRISPR-Cas13 is limited to targeting RNA and cannot interact with DNA. This restriction confines its application in nucleic acid detection to RNA, whereas CRISPR-Cas12 can detect both RNA and DNA through a straightforward reverse transcription step. These advantages position CRISPR-Cas12 as a vital tool for gene editing and nucleic acid detection. Further engineering efforts aimed at expanding the PAM recognition range and optimizing gene targeting specificity—such as through the directed evolution of Cas proteins and crRNA optimization—will further enhance the applications of CRISPR-Cas12 alongside CRISPR-Cas9 and CRISPR-Cas13 across various fields.

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Author contributions

Conceptualization, F. Y.; writing, M. X and F. Y.; visualization, M. X.; supervision, F. Y.

Declaration of interests

The authors declare no competing interests.

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