Single-Nucleotide mutations in FMR1 reveal novel functions and regulatory mechanisms of the fragile X syndrome protein FMRP

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Introduction

Fragile X syndrome (FXS) is a common cause of inherited intellectual disability and autism spectrum disorder (ASD). The prevalence of FXS is ~1 in 5000 males and 1 in 8000 females, and it accounts for 1–2% of all intellectual disability. The hallmark feature of FXS is intellectual disability, although there are other features for this disorder, such as macroorchidism, hyperflexible joints, and seizures, which are present in a subset of patients. The severity of intellectual disability ranges from mild to severe, where males are typically more severely affected than females because of the X-linked nature of the disorder. Additionally, patients often display characteristics associated with ASD, such as lack of eye contact, stereotypic behaviors, and social and language impairment, making it one of the most common known genetic causes of ASD to date.

FXS results from the inactivation or dysfunction of a single gene, FMR1. In a vast majority of patients, FMR1 is silenced by the expansion of an unstable triplet CGG-repeat motif in the 5′UTR that occurs in the maternal germ line. The number of CGG repeats is polymorphic in the general population, where 5–45 repeats are typical. Alleles with 45–200 repeats are referred to as premutation, as these alleles often experience a CGG-repeat expansion mutation and give rise to the alleles known as full mutation in the offspring (>200 CGG repeats). An FMR1 gene containing >200 repeats triggers an epigenetic event whereby the entire promoter region and flanking areas become hypermethylated and adopt a heterochromatin conformation, leading to the silencing of transcription and the absence of FMRP, the FMR1 gene product.

FMRP is a selective RNA-binding protein (RBP) that inhibits the translation of its mRNA targets (although not in all cases) and is most highly expressed in the brain. Upon specific neuronal activity, such as activation of the metabotropic glutamate receptor (mGluR) signaling pathway, protein synthesis at the synapse rapidly increases, including the synthesis of FMRP. Many genes bound by FMRP participate in modulating synaptic plasticity, a phenomenon widely believed to be the basis of learning and memory. A consequence of FMRP's absence is dysregulated translation of its target mRNAs, especially in response to neuronal signaling, which impedes synaptic plasticity and likely leads to the intellectual disabilities in patients. This model is supported by the significant level of correlation between FMRP targets and genes implicated in ASD and intellectual disability. Thus, FMRP is critical to proper translational regulation in the brain, particularly with regard to the molecular response to neuronal activity, and plays a major role in cognitive development.
Advances in high-throughput sequencing technology have made whole gene sequencing screens in a large, economical, and feasible patient set. Several groups have leveraged these new sequencing techniques to investigate point mutations or small indels in \textit{FMR1} as the potential causes of undiagnosed intellectual disability in patients. This review summarizes recent sequencing studies focused on the \textit{FMR1} gene and the variants discovered in these reports. We discuss the molecular impact of these variants and how several variants have revealed the novel functions of FMRP and \textit{FMR1} regulation.

**Canonical FMRP Functions**

To understand the impact that each variant has on FMRP, the known functions and molecular phenotypes caused by the absence of the protein must be discussed, as these were tested through several experiments to characterize the level of dysfunction. FMRP is most highly expressed in the brain, although it is present to a lesser degree in most other tissues.\footnote{21} It binds RNA through several different structural domains, such as the K-homology (KH) domains and an arginine/glycine-rich motif (RGG box) (Fig. 1). Other functional domains within FMRP are the two Agnet domains, which mediate protein–protein interactions, and the nuclear localization and export signals, which allow FMRP to shuttle between the nucleus and cytoplasm.\footnote{1} To date, much of the research regarding the functions of FMRP has focused on the RNA-binding properties, particularly as related to translational regulation in the postsynaptic space. Assays to determine the ability of FMRP to bind RNA are a common way to test for proper functioning of the protein.

FMRP is most abundant in the cytoplasm and neurons, which localizes to two main subcellular cytoplasmic compartments: the pre- and postsynaptic spaces of a neuronal connection. The main function of FMRP in the postsynapse, the more frequently studied location of FMRP function, is translational suppression of bound mRNAs. One of the ways that FMRP suppresses translation is through its association with dendritic polyribosomes where it stalls ribosomal translocation, thereby arresting protein synthesis of bound mRNAs.\footnote{19} After postsynaptic neuronal excitation, FMRP is quickly dephosphorylated and dissociates from its mRNA ligands, allowing rapid translation in response to the stimulus to facilitate synaptic plasticity.\footnote{22,23} The absence of FMRP uncouples the translation of its mRNA targets and neuronal signaling by allowing synthesis of these targets to occur unchecked, which likely impairs mGluR-mediated long-term depression, a major mechanism in modulating synaptic plasticity that relies on tightly controlled protein expression.\footnote{24,25} The ability of FMRP to respond to neuronal stimulation can be tested in cultured neurons using the synthetic glutamate analog (RS)-3,5-dihydroxyphenylglycine, which binds glutamate receptors and mimics neuronal activity via the mGluR pathway.

In addition to dysregulated protein translation at the synapse, another molecular consequence of lacking FMRP is exaggerated, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor endocytosis.\footnote{26} AMPA receptors are transmembrane molecules that bind the neurotransmitter glutamate to help propagate a synaptic transmission in excitatory neurons.\footnote{27} By regulating how many of these receptors are available at the surface of a synapse, cells can control the

![Figure 1. Schematic representation of \textit{FMR1}/FMRP at the DNA, mRNA, and protein levels. Each of the variants described in the text are shown by red arrows at the level where they are thought to cause FMRP dysfunction or misregulation. At the DNA level, vertical bars/boxes are the exons of \textit{FMR1}, with the unfilled white areas representing the UTRs, and the black horizontal lines represent intronic sequences. The beige box in the mRNA shows the coding sequence, which codes for FMRP. The green barrels at the protein level represent known functional domains within FMRP.](image-url)
strength and length of synaptic signaling, and the absence of FMRP shifts the balance to the increased internalization of the AMPA receptors. Although the exact mechanism by which FMRP regulates AMPA receptor trafficking is still being elucidated, assessing AMPA receptor internalization is a sensitive indicator of FMRP postsynaptic functioning. All together, these well-characterized activities of FMRP are tested in the presence of the variants that will be discussed as an indicator of the molecular effect each variant has on FMRP functionality.

**FMR1 Variants Inform of Novel FMRP Functioning and Regulation**

The *FMR1* gene was discovered in 1991 and has since been studied extensively. A number of deletions that encompass the whole gene or parts of the 5′ end have been described (see Coffee et al. for a comprehensive list). However, despite some previous investigation for mutational mechanisms other than the CGG-repeat expansion, very few point mutations have been shown to cause FXS. Until recently, only a single missense mutation in the coding region, known as the I304N mutation, had been found to cause FXS. This mutation is within the RNA-binding KH2 domain of FMRP, and the resulting FMRP is a functional null. The overwhelming lack of coding mutations led researchers to the conclusion that virtually all cases are caused by the CGG-repeat expansion and far less frequently by large deletions. Because of this belief, and the previous technical challenges and cost of sequencing an entire gene in a large number of individuals, the sequencing of *FMR1* in search of pathological point mutations is rarely performed even if clinical clues suggest an FXS-like disorder. However, with the recent advances in sequencing technology and the dramatic reduction in cost, sequencing has become more practical. In the largest study to date, the sequencing of *FMR1* in nearly 1000 developmentally delayed males with normal CGG-repeat lengths was performed in an attempt to identify novel, potentially causative variants. Additionally, several smaller *FMR1* sequencing studies have identified pathological point mutations as well. Overall, these studies detected a number of variants of interest, both coding and noncoding, which were functionally characterized and found to impair FMRP expression or function via several different mechanisms. Unexpectedly, several of these variants have uncovered the previously unknown functions of FMRP and the way that its expression is regulated.

**Coding Region Variants**

**G266E—missense mutation.** The *FMR1* missense variant c.797G>A, which causes a glycine-to-glutamate change at amino acid 266 (p.G266E), was discovered in a patient referred to clinicians because of developmental delay, along with multiple other behavioral and physical features commonly associated with FXS. The patient was tested for the typical repeat expansion mutation, which was found to be within the normal size range. Several other molecular diagnostic and imaging tests for other disorders and syndromes were negative as well, leaving the cause of the patient’s phenotype unresolved. Because of the numerous FXS characteristics displayed by the patient, sequencing of the *FMR1* gene was performed, revealing the G266E variant. The patient’s immediate family was also sequenced, showing that the variant was inherited from his unaffected mother, and all three of his unaffected brothers did not have the variant allele.

To determine whether this was a benign or pathological mutation, several different assays were performed to test the functionality of the variant, FMRP. The ability of the G266E-FMRP to bind RNA was tested through immunoprecipitation assays, where an antibody was used to purify FMRP from cell lysates and then quantitative reverse transcription polymerase chain reaction was used to detect the copurified mRNAs that were bound to FMRP. This experiment revealed that the G266E-FMRP was not binding several known mRNA targets (*Map1B*, *PSD-95*, and *CamKII*), suggesting that the mutation disrupts the RNA-binding function. Using *Fmr1* KO neurons in culture and introducing various forms of FMRP, AMPA receptor endocytosis was assessed. When wild-type (WT) FMRP was expressed in KO neurons, the increased AMPA receptor internalization phenotype typically found in KO neurons was rescued back to normal levels. The expression of G266E-FMRP in KO neurons, however, did not produce any level of rescue of AMPA receptor internalization. Finally, the ability of FMRP to associate with polyribosomes was examined. Sucrose gradients were used to isolate different polyribosome fractions from *Fmr1* KO mouse embryonic fibroblasts, expressing exogenous WT or G266E-FMRP, and then probed for the presence of FMRP in each fraction by Western blotting. If WT FMRP is in all polyribosome fractions as expected, the G266E-FMRP does not associate with polyribosomes at all, suggesting that this FMRP variant is a functionally null protein with regard to translational regulation.

In conjunction with these functional assays, the authors studied the evolutionary conservation and structural consequences of the glycine-to-glutamic acid mutation. The position is highly conserved with regard to both KH domain amino acid content in general and throughout the evolution of FMRP. Structurally, position 266 likely requires a small, flexible, and nonpolar amino acid, all the characteristics of glycine. Glutamic acid, on the other hand, is large and negatively charged and is predicted to clash sterically and ionically with surrounding amino acids. Together, these data show that several canonical functions of FMRP and its structure are disrupted by the G266E mutation, which represents the second reported mutation of pathological coding region in *FMR1*.

**R138Q—missense mutation.** The *FMR1* missense variant c.413G>A, resulting in an arginine-to-glutamine change at the protein level of position 138 (p.R138Q), was identified in a male patient who was originally referred to Emory...
Genetics Laboratory for CGG-repeat molecular testing, which was found to be within the normal range. His DNA sample was later included in the FMR1 sequencing screen by Collins et al., where the variant was discovered. The patient is mildly intellectually disabled and developmentally delayed and has had intractable seizures, a trait that is present in 10–20% of FXS patients. As with the G266E variant, R138Q-FMRP was tested for its ability to bind RNA, associate with polyribosomes, and rescue exaggerated AMPA receptor internalization. Surprisingly, in each of these assays, R138Q-FMRP activity was normal, suggesting that these FMRP functions are preserved in the presence of the variant. Because each of these assays tests for a functionality that is typically carried out by FMRP in the postsynaptic space, the authors decided to examine whether several known presynaptic functions of FMRP were intact. First, they examined the neuromuscular junction (NMJ) in Drosophila, where dfmr1-deficient Drosophila shows an overgrowth phenotype. Additionally, because there is no neural postsynapse at the NMJ, this effectively allows for the investigation of FMRP’s presynaptic functions in isolation. Interestingly, in dfmr1-deficient Drosophila, the introduction of a WT dFmrp rescued the overgrowth phenotype, whereas the R140Q-dFmrp (the amino acid of interest is at position 140 in Drosophila) could not, suggesting some type of presynaptic dysfunction.

To show that this presynaptic defect exists in a mammalian neural setting, the group utilized electrophysiological assays in mouse hippocampal and cortical tissue slices to examine the action potential (AP) duration, which has previously been shown to be lengthened in Fmr1 KO tissues. This AP lengthening phenotype could be rescued by delivering an amino terminal FMRP fragment consisting of amino acids 1–298 (FMRPΔ298) to the presynapse. Using this paradigm, KO brain tissue was isolated, and a version of FMRP that included the R138Q mutation (R138Q-FMRPΔ298) was introduced to the system to determine its ability to rescue the AP phenotype. In contrast to FMRPΔ298, the R138Q-FMRPΔ298 variant was unable to reduce the AP lengthening in hippocampal and cortical CA3 pyramidal neurons, providing further evidence of a presynaptic dysfunction of FMRP caused by the R138Q mutation. Importantly, this presynaptic defect is likely unrelated to the typical FMRP function of translational regulation because of previous findings, as well as the fact that FMRPΔ298 is missing in the KH2 domain required for polyribosome association and because of the relatively quick rescue effect after adding FMRPΔ298. A possible reason for the observed AP lengthening phenotype was identified through protein–protein association assays. FMRPΔ298 has previously been shown to bind the BK (Big Potassium) channels, particularly the β4 subunit of the presynaptic membrane, an important structure in regulating the release of neurotransmitters and neuronal excitability. Using coimmunoprecipitation assays, the authors discovered that the interaction between FMRP and the BK-β4 subunit was severely impaired by the R138Q mutation.

Taken together, these results reveal a novel presynaptic function of FMRP that is independent of RNA-binding and translational regulation. Furthermore, because the patient suffers from seizures, as do a subset of FXS patients, the R138Q variant may reveal mechanistic insights into this phenotype and serves as a starting point for studying how the lack of FMRP can result in seizures.

C.1457insG—frameshift with early termination. An insertion of a guanine in exon 15 of FMR1 (c.1457insG) was discovered in a patient from a sequencing screen of 16 male patients with intellectual disability, autistic behaviors, impaired social interaction, and at least one physical characteristic typically associated with FXS, but normal CGG-repeat lengths. This single-nucleotide insertion is predicted to cause a frameshift that adds 22 novel amino acids downstream of the mutation and disrupts the RGG box domain, followed by a premature termination codon that would truncate the protein (p.G538fs*23). A lymphoblastoid cell line was derived from the patient and used to determine the levels of FMR1 and FMRP, both of which were significantly reduced compared to healthy controls. In addition to a reduction in quantity, Western blotting analysis showed that the patient’s FMRP that was present was smaller in size, confirming the predicted truncation due to the introduction of the early stop codon. Interestingly, when the authors examined the localization of this truncated FMRP, they found that some or most of the proteins were present in the nucleus, specifically the nucleolus, of various cell types as opposed to the typical cytoplasmic localization of FMRP. Computational analysis of the novel 22 amino acid sequence revealed a motif similar to known nuclear localization signals (NLSs), suggesting a potential gain of function caused by the frameshift mutation. The authors leveraged this unusual finding in a series of mutational experiments carried out in cultured cells and Drosophila models, which validated the novel amino acid sequence as a functional NLS. However, this NLS could not localize FMRP to the nucleus in the presence of an intact C-terminal end of FMRP, suggesting that the C-terminus contains a nuclear export domain that is dominant to the novel NLS. Indeed, FMRP was only retained in the nucleus when both the C-terminus was deleted and novel NLS was present. This finding likely underlies the patient’s disabilities, as this was the only mutational combination that did not cause an axonal misguidance phenotype known to occur in a subset of Drosophila neurons when dFmrp is overexpressed. These findings are supported by previous data which showed that FMRP remains cytoplasmic with the deletions of the C-terminus through the RGG box, indicating the importance of the novel amino acid sequence as the driver of the mislocalization.

Overall, the patient’s disabilities are likely due to the addition of a functional NLS and the concomitant loss of the C-terminal nuclear export signal as an opposing localization...
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factor. This combination appears to mediate the retention of FMRP in the nucleus, preventing it from performing its various tasks at the synapse. Further research to more precisely identify the amino acid sequence at the C-terminal end of FMRP that constitutes the nuclear export domain would provide a greater understanding of the localization mechanisms that govern FMRP shuttling.

**S27X—nonsense mutation.** A mutation in exon 2 of FMR1 (c.80C>A) was discovered in a male patient displaying classic FXS features, such as intellectual disability, common facial dysmorphism, and macroorchidism. Additionally, the patient suffered from epilepsy, autistic features and showed very little use of language. The mutation was found to be transmitted by his mother, who was heterozygous and suffered from mild intellectual disability and several behavioral deficits. This mutation is predicted to change a serine to an early termination codon (p.S27X) and lead to a severely truncated FMRP.

Southern blotting showed that both the proband and his mother had CGG repeats within the normal range. However, FMRP was undetectable in a lymphoblastoid cell line of the proband, suggesting that either the mRNA message is degraded by the cell via the nonsense-mediated decay (NMD) pathway before translation can occur or it is not amenable to the type of analysis used (Western blotting) because it is such a highly truncated version of the protein. Further studies to determine the presence or absence of FMR1 mRNA in the patient would help identify the mechanism underlying the lack of FMRP. Additionally, the assessment of the mother’s FMR1 and FMRP levels would be useful because her X-inactivation pattern was tested and found to be equally distributed in blood, which suggests that about half of normal levels of FMRP should be detectable, although compensatory mechanisms may factor into the total level of FMRP expression. An analysis of FMR1 mRNA levels in the mother would help elucidate whether the NMD pathway is degrading the variant transcript as approximately half of the FMR1 produced would have the mutation. In either case, the resulting FMRP in the proband, if any, is so severely truncated that it would almost certainly be a null allele. Thus, because of the severe truncation/absence of FMRP and the FXS features displayed by the proband, this mutation is likely to be cause of the patient’s deficits.

### Untranslated Region Variants

**5’UTR—c. -332G>C, c. -293T>C, and c. -254A>G.**

Three promoter variants of interest were detected in the study by Collins et al, each found once in different patients, not found in any control individuals, and all conserved throughout mammalian evolution. The most upstream variant, c. -332G>C, is located within a putative-binding motif for the transcription factors Sp1 and AP-2, both of which bind GC-rich motifs to modulate transcription. Another variant identified, c. -293T>C, is within or adjacent to three different functional sequence motifs: the FMR1 transcription start site II, an initiator-like sequence, and a TATA-like sequence, suggesting that it may interfere with transcription initiation. The third patient-associated variant identified, c. -254A>G, is near the primary transcription start site and resides within an initiator-like sequence as well. Reporter assays were used to determine whether any of the variants had an impact on the expression of FMR1. Luciferase vectors were constructed to include the FMR1 promoter upstream of the firefly luciferase gene with each of the three variants, which were transfected into HeLa cells to determine expression levels. All three variants significantly decreased the amount of reporter expressed compared to a control, ranging from ~6% to 36% of normal expression. Although more thorough analyses are required to definitively characterize these variants as pathological, these data suggest that the initiation of FMR1 transcription may be hampered by these variants and could result in the reduced levels of functional FMRP.

**3’UTR—c.*746T>C.** Several 3’UTR variants were identified in the sequencing screen by Collins et al, one of which, c.*746T>C, was studied extensively. This variant was identified in six unrelated male patients and none of the control individuals tested. Both the nucleotide and motif are highly conserved as evidenced by the PhyloP (2.76), GERP (5.52) and PhastCons (1.0) scores. One of these patients was clinically evaluated at Emory Genetics Laboratory, was found to have moderate intellectual disability (Stanford–Binet Intelligence Scale IQ of 47), was nonverbal, and was delayed in achieving several physical milestones, such as sitting and walking (Suhl et al, in press www.pnas.org/cgi/doi/10.1073/pnas.1514260112). He had also been previously diagnosed with ASD and attention deficit hyperactivity disorder.

Because 3’UTRs are typically associated with the regulation of translation, the authors first tested whether the expression of a reporter gene was altered by the variant. Using luciferase vectors containing the full length FMR1 3’UTR from a patient or a normal individual, a significant decrease in reporter activity compared to a control 3’UTR was detected. These data were supported by a lymphoblastoid cell line derived from the patient, where endogenous FMRP was reduced by ~20% compared to two control lymphoblastoid lines. Although statistically significant, a modest reduction in FMRP such as this may not have a severe developmental impact. Therefore, the function of the variant in a neuronal context under steady-state and stimulatory conditions was investigated. Interestingly, reporter assays in mouse primary cortical neurons in culture revealed not only a reduction in steady-state expression but also a lack of response to glutamate signaling, a critical feature of FMRP function in the postsynaptic space. To determine a mechanism underlying the observed deficits in reporter activity, gel shift and immunoprecipitation assays were used to test the hypothesis that the variant disrupted the interaction of an RBP. Through these assays, a dosage-sensitive and specific protein interaction with the FMR1 c.*746 locus was detected and subsequently identified as HuR through mass
spectrum and gel supershift assays. HuR is a ubiquitously expressed RBP that binds U-rich motifs nearly identical to the c.746 locus to increase transcript stability and promote translation. Importantly, binding assays revealed that the patient allele impaired the binding of HuR, which may be the cause of the deficits observed in the reporter assays. In support of this, cross-linking immunoprecipitation (CLIP) assays, which identify sites of RNA/protein interactions in vivo, showed that HuR does indeed bind several sites in the FMR1 3′UTR in nonneuronal cells, including c.746 locus. Other CLIP experiments showed that other members of the Hu protein family, the neuronally expressed HuB, HuC, and HuD, bind the locus in mouse brain tissue, suggesting that all members interact with, and likely regulate, FMR1 in a variety of tissues.

These findings implicate the 3′UTR as mediating activity-dependent translation of FMR1 at the synapse. Additionally, this finding is in line with the previous data which showed that even with preexisting FMRP at the synapse, local synthesis of FMRP in response to neuronal activity is critical to proper synaptic function. Finally, these results underscore the importance of annotating and empirically testing variants in the noncoding portions of genes, as these can influence gene expression and regulation and may ultimately lead to a clinical phenotype.

Conclusions
The studies covered here all suggest that variants within the FMR1 gene other than the CGG-repeat expansion mutation can cause dysfunction of FMRP. Similar to the I304N mutation, the G266E mutation is within a conserved amino acid in a KH domain and is very likely to be responsible for the patient’s intellectual and behavioral disabilities as all of the well-studied functions of FMRP are deficient. The S27X mutation is also very likely to be the root of the patient’s symptoms because the truncation is so severe and FMRP is absent in a cell line derived from the patient. The other variants, however, will require more investigation to definitively classify them as pathological because FMRP is still present to some degree, most were detected in only one individual, and each represents novel functions or regulatory mechanisms of the gene that have not been extensively evaluated. Several avenues of research would help validate these as truly causative of disease. One approach would involve generating mouse models for each variant to test whether there are observable phenotypes and/or molecular defects that remain in the context of an entire animal and in the appropriate tissue (ie, brain), as opposed to in vitro and cell-based assays. In the case of the c.746T>C variant, the only variant identified in more than one person with developmental delay, more patients with uncharacterized intellectual disability could be genotyped relatively quickly and economically at this locus to better determine the frequency and enrichment of the variant in a larger number of affected individuals and confirm its segregation with intellectual disability/ASD.

Several approaches to treat FXS have been considered, such as the reactivation of the full mutation allele by demethylating or chromatin modifying drugs and targeting neuronal proteins affected by the loss of FXS, such as the PI3K and mTOR. However, the primary therapeutic approach for the treatment of FXS is currently aimed at dampening signaling of the mGluR pathway by antagonizing these receptors with small molecules. The hypothesis is that the translation of genes in the absence of FMRP that help to mediate synaptic plasticity is impaired because of the lack of FMRP as a negative regulator. Reducing the amount of mGluR activation by blocking the receptors with an antagonist may help restore the appropriate translational status of various genes within the postsynaptic space. However, at least some of the variants described in this review may require a different therapeutic approach because the mechanisms of dysfunction differ from lacking FMRP entirely. For example, the R138Q mutation selectively impairs presynaptic functions of the protein, which means treatment plans that involve reducing postsynaptic mGluR signaling may not be effective. More research is required for each of these specific cases and, more broadly, in identifying additional FMR1 point mutations. If more of these nonrepeat mutations in FMR1 are found to cause disease and as whole gene-and-exome sequencing becomes more affordable, it may be useful to sequence FMR1 in cases of undiagnosed developmental delay where the CGG repeat is of typical length. Additionally, it may also be useful to initiate a related, but separate, clinical classification that differs from FXS but is FMR1-driven, which would include these types of noncanonical mutations that have different mechanisms of action from the repeat expansion-driven pathology.

Author Contributions
Conceived the concepts: JAS and STW. Analyzed the data: JAS and STW. Wrote the first draft of the manuscript: JAS and STW. Contributed to the writing of the manuscript: JAS and STW. Agree with manuscript results and conclusions: JAS and STW. Jointly developed the structure and arguments for the paper: JAS and STW. Made critical revisions and approved final version: JAS and STW. All authors reviewed and approved of the final manuscript.

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