Candidate Heterotaxy Gene FGFR4 Is Essential for Patterning of the Left-Right Organizer in Xenopus

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Congenital heart disease (CHD) is the most common birth defect, yet its genetic causes continue to be obscure. Fibroblast growth factor receptor 4 (FGFR4) recently emerged in a large patient exome sequencing study as a candidate disease gene for CHD and specifically heterotaxy. In heterotaxy, patterning of the left-right (LR) body axis is compromised, frequently leading to defects in the heart's LR architecture and severe CHD. FGF ligands like FGF8 and FGF4 have been previously implicated in LR development with roles ranging from formation of the laterality organ [LR organizer (LRO)] to the transfer of asymmetry from the embryonic midline to the lateral plate mesoderm (LPM). However, much less is known about which FGF receptors (FGFRs) play a role in laterality. Here, we show that the candidate heterotaxy gene FGFR4 is essential for proper organ situs in Xenopus and that frogs depleted of fgfr4 display inverted cardiac and gut looping. Fgfr4 knockdown causes mispatterning of the LRO even before cilia on its surface initiate symmetry-breaking fluid flow, indicating a role in the earliest stages of LR development. Specifically, fgfr4 acts during gastrulation to pattern the paraxial mesoderm, which gives rise to the lateral pre-somite portion of the LRO. Upon fgfr4 knockdown, the paraxial mesoderm is mispatterned in the gastrula and LRO, and crucial genes for symmetry breakage, like coco, xnr1, and gdf3 are subsequently absent from the lateral portions of the organizer. In summary, our data indicate that FGF signaling in mesodermal LRO progenitors defines cell fates essential for subsequent LR patterning.

Keywords: FGF signaling, left-right patterning, gastrulation, Xenopus, congenital heart disease, heterotaxy

INTRODUCTION

Left-right (LR) asymmetry is a major characteristic of the vertebrate body plan. While externally symmetric, chordates display a specific internal LR arrangement of their visceral organs. In patients with heterotaxy, LR development is defective and organs are mispatterned relative to the LR axis, which often results in compromised LR architecture of the heart and clinically severe cardiac dysfunction. Although heterotaxy is a predominantly genetic disease, causal genes remain largely unidentified (Zaidi and Brueckner, 2017). Exome sequencing of congenital heart disease (CHD) patients identified three individuals with damaging mutations in fibroblast growth factor receptor 4 (FGFR4). The first patient, with hypoplastic left heart syndrome, had a de novo mutation (Asp297Asn). The second patient, with an L-transposition of the great arteries and tricuspid atresia, had an inherited stopgain at Gly705. And finally, a third patient had an inherited damaging splice mutation associated with a hypoplastic main pulmonary artery and a left superior vena cava that...
emptied into the coronary sinus (Zaidi et al., 2013; Jin et al., 2017). The patients’ phenotypes suggest defects in LR patterning consistent with an established role for FGF signaling in LR patterning. We sought to investigate this further by studying the role of FGFR4 in the LR patterning cascade.

FGF signaling is governed by an array of FGF ligands and their receptors (FGFRs). In mammals, 18 FGFs and four FGFRs (FGFR1-4) are employed in a broad variety of developmental processes, including LR patterning (Teven et al., 2014). LR patterning begins at the left-right organizer (LRO) which forms in the posterior mesoderm at the end of gastrulation. At the LRO, cilia beat to create leftward extracellular fluid flow that breaks bilateral symmetry (Essner et al., 2002; Schweickert et al., 2007; Babu and Roy, 2013; Yoshida and Hamada, 2014). For LR patterning to occur properly, LRO progenitor cells must be specified in the mesoderm, and the LRO has to be physically formed and properly engage in ciliogenesis and cilia signaling. Once the flow signal is detected in the left margin of the LRO, it is transmitted via nodal signaling to the left lateral plate mesoderm (LPM), inducing asymmetric pitx2c expression, which then results in asymmetric organogenesis (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshika et al., 1998; Campione et al., 1999; Kawasaki et al., 2011). FGF signaling plays a role in several steps in this process.

Zebrafish homozygous for a stopgain mutation of FGF8 have been shown to physically lack a LRO (Albertson and Yelick, 2005). On the other hand, FGF8 acts differentially in chick, mouse, and rabbit to regulate asymmetric gene expression at the LRO and LPM (Boettiger et al., 1999; Meyers and Martin, 1999; Fisher et al., 2002). In contrast, FGF4, stimulates ciliogenesis in the zebrafish LRO, but is not required to physically form the LRO and LPM (Campione et al., 1999; Kawasumi et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999; Kawasaki et al., 2011). FGF4 signaling plays a role in several steps in this process.

Materials and Methods

Xenopus

Xenopus tropicalis were housed and cared for in our aquatics facility according to established protocols that were approved by the Yale IRB—Institutional Animal Care and Use Committee (IACUC). Embryos were produced by in vitro fertilization and raised to appropriate stages in 1/9X MR as per standard protocol (del Viso and Khokha, 2012).

CRISPR Injection and Validation

Injections of Xenopus embryos were carried out at the one-cell stage using a fine glass needle and Picospritzer system, as previously described (del Viso and Khokha, 2012). Small guide RNAs (sgRNAs) containing the following fgfr4 target sites were designed from the v7.1 model of the X. tropicalis genome: CRISPR-1 (exon 3): 5'-AGGAAACTTTGCTGCGGAG-3', CRISPR-2 (exon 6): 5'-AGTGTGTTCCCATCAAGAGTTG-3', and CRISPR-3 (in exon 8): 5'-TGCAGGGGATACACATGTCTGG-3'. One-cell embryos were injected with 1.5 ng Cas9 Protein (PNA-Bio) and 400 pg of targeting sgRNA and raised to desired stages as previously described in detail (Bhattacharya et al., 2015). For genotyping, F0 embryos were raised to stage 45 and lyzed in 50 mM NaOH as previously described (Bhattacharya et al., 2015). Editing by CRISPR-1 was verified by amplifying from tadpole genomic DNA an 800 bp fragment around the prospective cut site in fgfr4, Sanger sequencing and subsequent ICE (Inference of CRISPR Edits) analysis with Synthego software (Hsiau et al., 2018). The efficacies of CRISPR-2 and -3 were assessed by amplifying a ~1 kb fragment around the prospective cut sites and performing the T7 Endonuclease assay (Guschin et al., 2010). For this, PCR products were denatured and re-annealed, and mismatches between re-annealed wildtype and CRISPR-edited sequences were detected by T7 Endonuclease I digest (NEB). Digests were visualized on 2% agarose gels. The following primers were used to produce PCR products containing the prospective cut sites:

| sgRNA | TARGET SITE (5'-3') | FORWARD PRIMER | REVERSE PRIMER |
|-------|---------------------|----------------|----------------|
| 1     | AGGAACG TTTGC      | CTTG  | TCTGCTT        |
|       | TGGCCG GAAGGG AGTGTG | CTCAGTGA | CTCACCTGG |
| 2     | ACTGT TGCAGG        | CAAGTCC | CAGCCAT |
| 3     | ATACACAT GTCTGG  | GCGTGTG | AGTTTGCT |

Cardiac Looping

Stage 45 Xenopus tadpoles were paralyzed with benzocaine and scored under a stereomicroscope. Looping was determined by position of the outflow tract. D-loops were defined as outflow tracts directed to the right, and L-loops to the left.

In situ Hybridization

Digoxigenin-labeled antisense probes for pitx2 (TNeu083k20), cocom (Tegg007d24), xnr1 (TGas124h10), gdf3 (Tgas137g21), gsc (TNeu077f20), xnr3 (Tgas011k18), foxf1 (Tneu058M03), vent2 (Tgas007d24), and nodal (Tgas007d24) were designed and synthesized (GeneSolutions, Inc.) and used for whole mount in situ hybridization.
(BG885317), myf5 (TGas127b01), xbra (TNeu024F07), fgfr4 (Dharmacon, Clone ID: 7521919) were in vitro transcribed using T7 High Yield RNA Synthesis Kit (E2040S) from New England Biolabs. Embryos were collected at the desired stages, fixed in MEMFA for 1–2 h at room temperature (RT) and dehydrated in 100% ethanol. GRPs were dissected post fixation prior to dehydration. Briefly, whole mount in situ hybridization of digoxigenin-labeled antisense probes was performed overnight, the labeled embryos were then washed, incubated with anti-digoxigenin-AP Fab fragments (Roche 11093274910), and signal was detected using BM-purple (Roche 11442074001), as previously described in detail (Khokha et al., 2002).

**GRP Immunofluorescence**

*Xenopus* embryos were collected at stage 17 and fixed for 2 h at RT in 4% paraformaldehyde/PBS. All samples were washed three times in PBS + 0.1% TritonX-100 (PBST) before incubating in PBST + 3% BSA blocking solution for 2 h at RT. Samples were then placed in blocking solution + primary antibody ON at 4°C. Samples were washed three times in PBST before incubating in blocking solution + secondary antibody/Phalloidin for 2 h at RT. Samples were washed three times in PBST and one time in PBS before mounting in Pro-Long Gold (Invitrogen) and imaging on a Zeiss 710 confocal microscope. Primary antibodies and dilutions for IF: Mouse monoclonal anti-acetylated tubulin, clone 6-11B-1, SIGMA Catalog: T-6793 (1:1000); rabbit polyclonal anti-MYOD (aa-1-150), LSBio, Catalog: LS-C143580 (1:200). Alexa488 and 594 conjugated anti-mouse and rabbit secondary antibodies were obtained from Thermo Fisher Scientific and used at a 1:500 dilution. Alexa647 phalloidin (Molecular Probes, 1:50) was used to stain cell boundaries.

**Quantifications**

All quantifications were done using standard Student’s t-tests and taking into account three or more replicate experiments. To measure GRP area, the GRP area was outlined based on cell morphology and measured in ImageJ. To measure the presomitic area of the GRP, the GRP was first outlined based on morphology and the myoD positive portion of this area was subsequently outlined and similarly measured. Cilia were counted by thresholding individual acetylated-tubulin immunostaining images in ImageJ and performing particle analysis. The cilia count was then additionally verified by manual counting. Embryo numbers: N = 15–25 for total GRP and PSM area and N = 10 for cilia numbers. Embryo numbers for phenotype quantification and in situ hybridization experiments are indicated in each figure.

**RESULTS**

**Fgfr4 Is Required for GRP Patterning**

To investigate whether *FGFR4* plays a role in LR development, we performed F0 CRISPR editing of the gene in *X. tropicalis*. This strategy effectively introduces damaging mutations in both alleles within 2 h after microinjection, and F0 tadpoles can be scored for organ situs 3 days later by simple inspection. We have found that known gene knockdown phenotypes can be replicated using F0 CRISPR editing in 9 out of 10 cases (Bhattacharya et al., 2015). In vertebrates, the cardiac tube initially forms in the midline, but then normally loops to the right (D-loop). Looping to the left (L-loop) or remaining midline (A-loop) is abnormal and suggests LR patterning defects consistent with heterotaxy (Baker et al., 2008; de Campos-Baptista et al., 2008; Rohr et al., 2008). Similarly, the gut depends on correct LR patterning to become coiled counter-clockwise and the intestinal rotation can be inverted in heterotaxy (Campione et al., 1999).

We generated three sgRNAs independently targeting three non-overlapping sites in exons 3 (CRISPR-1), 6 (CRISPR-2), and 8 (CRISPR-3) of *fgfr4*, respectively. We verified gene modification using PCR amplification of the cut site followed by either Sanger sequencing and ICE (Inference of CRISPR Edits) analysis or the T7 Endonuclease I assay (Supplementary Figure 1). F0 CRISPR for all three sgRNAs led to tadpoles with cardiac L-loops and inverted gut looping (Figures 1A–C), indicating that *fgfr4* plays a role in establishing organ laterality.

Next, we tested if *fgfr4* depletion affected global LR patterning. Heart and gut looping both depend on activation of asymmetric gene expression in the left LPM prior to heart and gut tube morphogenesis (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999). *Pitx2c*, a homeobox transcription factor, is normally expressed in the left LPM. In a third of *fgfr4* CRISPR animals, we found *pitx2c* transcripts to be completely absent from the LPM, while a small minority displayed abnormal right-sided or bilateral *pitx2c* expression (Figures 1D,E). These results confirm that global LR development is compromised prior to organogenesis in *fgfr4*-depleted animals.
signal and is consistent with absence of pitx2c expression. The reduction/absence of gdf3 is also consistent with absent pitx2c, since gdf3 facilitates the transmission of the nodal signal to the LPM (Vonica and Brivanlou, 2007).

To further investigate the loss of these lateral nodal-related signals, we considered the possibility that the GRPs of fgfr4-depleted embryos were fundamentally mispatterned even prior to fluid flow. At stage 16, before cilia driven flow is established, coco, xnr1, and gdf3 transcripts are expressed mostly bilaterally in control embryos. Notably, all three transcripts were strongly reduced or absent (Figures 2A,C,E), indicating that the GRP is not patterned correctly in fgfr4-depleted embryos, irrespectively of fluid flow.

Abnormal patterning of the GRP suggests that its cellular composition may be affected. To assess GRP morphology, we performed F-actin/phalloidin stain and acetylated tubulin immunostaining to visualize cell boundaries and cilia, respectively (Figures 3A–H). LRO cells of the GRP normally form a teardrop structure composed of small mesodermal ciliated cells. GRPs of fgfr4 CRISPR embryos were morphologically distinct and composed of larger cells lacking cilia, resembling the neighboring endoderm (Figures 3A–D; phalloidin). Consistently, we measured a dramatically reduced total LRO area upon fgfr4 knockdown (Figure 3L). In mildly affected GRPs, the natural teardrop shape of the LRO was preserved, albeit reduced in area (Figures 3B,F), whereas more severe cases displayed only one or two rows of mesodermal cells and lacked the regular teardrop structure (Figure 3D,H). Because pre-somitic GRP markers coco, xnr1, and gdf3 were reduced or absent in fgfr4 CRISPR embryos, we used an antibody against the myogenic transcription factor myoD to visualize the pre-somitic GRP (Figures 3I–K). The pre-somitic mesoderm (PSM) protrudes into the gastrocoel between the hypochordal central portion of the GRP and the surrounding endoderm. We found the myoD-positive portion of the GRP to be notably reduced relative to total GRP area in CRISPR embryos (Figures 3I–K,M), indicating a specific loss of pre-somitic GRP, which is consistent with the reduction in coco, xnr1, and gdf3 expression. Finally, even though we counted fewer cilia per GRP in fgfr4 CRISPR embryos (Supplementary Figure 2B), we quantified a similar cilia per GRP area ratio to that of control embryos (Supplementary Figures 2A,C), suggesting that fgfr4 does not exert an effect on cilia differentiation per se, but rather affects the area of pre-somitic LRO. Altogether, this data suggests that fgfr4 is required for pre-somitic GRP patterning.

Fgfr4 Patterns the Paraxial Mesoderm During Gastrulation

Fgfr1 is expressed in the zebrafish LRO, where it activates essential ciliogenesis genes (Neugebauer et al., 2009). We
FIGURE 2 | GRP patterning is defective in fgfr4 CRISPR mutants. Most representative expression patterns of pre-somatic GRP markers coco, xnr1, and gdf3 in stage 17 (A,C,E) and stage 19 (B,D,F). GRPs of control and fgfr4 F0 CRISPR animals; ventral view of GRPs, anterior is to the top. Graphs show percentages of embryos with differential expression patterns of coco, xnr1, and gdf3. **p < 0.01, ***p < 0.001, ****p < 0.0001.

considered that fgfr4 may play a similar role in the GRP but were unable to detect fgfr4 transcripts in the GRP (Figures 4C,D). Expression in the developing somites, eyes, and LPM of neurula stage embryos (Figures 4A,B,D) served as a positive control for transcript detection. The GRP is composed of hypochordal and PSM and both these tissues are specified during early gastrulation from dorsally located superficial (SM) and paraxial/myogenic mesoderm, respectively (Hopwood et al., 1989, 1991; Zetser et al., 2001; Shook et al., 2004; Stubbs et al., 2008; Walentek et al., 2013). Because fgfr4 is expressed in the dorsal mesoderm during gastrulation (Figures 4E–H), we hypothesized that it could regulate early mesodermal patterning. In the early gastrula (stage 10), an array of markers is expressed in the mesoderm in a regionally restricted manner. We analyzed gene expression specific for the dorsal organizer (gsc, xnr3), paraxial/myogenic (myf5) mesoderm, superficial (foxj1) mesoderm, and ventral (vent2) mesoderm. Early gastrula (stage 10) fgfr4-depleted embryos showed intact patterning of the organizer, ventral, and superficial mesoderm but had absent myf5 expression in the paraxial/myogenic mesoderm (Figures 5A–F). Moreover, the pan-mesodermal marker xbra was normally expressed throughout the mesoderm of stage 10 fgfr4 CRISPR embryos, indicating that basic mesodermal identity was preserved even though myf5 expression was absent (Figures 5E,K). Toward the end of gastrulation (stage 12), myf5 expression was partially recovered, but markedly mispatterned (Figure 5H) and the upstream myogenic factor myoD was mispatterned in the same region (Figure 5I). The superficial mesoderm remained correctly patterned via foxj1 at this stage (Figure 5G). Interestingly, midline bisection of late gastrula fgfr4-depleted embryos revealed an additional reduction in xbra expression in the involuted mesoderm (Figures 5J,L), confirming a previously reported relationship between FGF signaling and mesodermal xbra expression (Isaacs et al., 1994). These results altogether suggest that fgfr4 is specifically required during gastrulation to pattern the paraxial/myogenic mesoderm and also maintain xbra expression in the involuted mesoderm. Loss of this paraxial mesoderm is then reflected in loss of lateral LRO markers coco, xnr1, and gdf3 which results in LR patterning defects.

DISCUSSION

In this study, we propose a role for the candidate heterotaxy gene FGFR4 in patterning the paraxial mesoderm, which
FIGURE 3 | GRP morphology and identity are altered in fgfr4 CRISPR embryos. (A–D) GRPs of fgfr4 CRISPR animals are morphologically distinct, as shown by phalloidin (actin) and anti-acetylated tubulin (cilia) stain; phenotypes ranging from mild (B) to severe (C,D), depending on loss of small mesodermal ciliated cells. (E–H) Higher magnification of GRPs shows loss of ciliated GRP area in fgfr4 CRISPR embryos (G, H). (I–K) The pre-somatic, myoD positive portion of the GRP (outlined) is drastically reduced in fgfr4 CRISPR embryos, even in embryos in which the overall GRP morphology is preserved (J). (L) Quantification of total GRP area, defined morphologically by small, ciliated cells, is reduced in fgfr4 CRISPR embryos. (M) The myoD positive area of the GRP, normalized to total GRP area, is specifically reduced in fgfr4 CRISPR embryos. Scale bars in (A–D, I–K) = 40 µm, in (E–H) = 20 µm. ** p < 0.01, *** p < 0.001.

FIGURE 4 | Fgfr4 expression during early X. tropicalis development, detected by in situ hybridization. (A,B) Whole stage 19 embryos; expression is detected in the head region (2: eyes), lateral plate mesoderm (3), anterior somites (1a) and posterior pre-somatic mesoderm (1b). (C,D) Transcripts were not detected in stage 16 and 19 GRPs (arrowheads). (E,F) Stage 10.5 embryos, whole (E, dorsal view) or bisected through the dorsal midline (F); fgfr4 is broadly expressed in the ectoderm (4) and dorsal marginal zone (5). (G,H) Stage 12 embryos, whole (G, dorso-vegetal view) or bisected (H); fgfr4 is absent from the marginal zone (5), but is expressed in the anterior migrating involuted mesoderm (6).

contributes to the formation of the lateral LRO. The PSM of the GRP of fgfr4 knockdown embryos lacks general myogenic patterning via myoD, but also coco, xnr1, and gdf3, which are specific markers for the PSM exposed to the gastrocoel and are indispensable for the GRP’s function as a LRO (Vonica and Brivanlou, 2007; Schweickert et al., 2010).

Both the hypochordal and PSM, which compose the GRP, are specified during early gastrulation from superficial (SM) and paraxial mesoderm, respectively. Multiple genes are known
FIGURE 5 | The paraxial myogenic mesoderm is mispatterned in fgfr4 CRISPR embryos. (A–F, G–J) Dorsal-vegetal views showing expression of an array of mesodermal markers in stage 10 (A–F) and stage 12 (G–J) embryos. Expression of paraxial mesoderm markers myf5 and myoD is perturbed in fgfr4 CRISPR embryos. (K, L) Xbra expression in embryos bisected through their dorsal midline at stages 10.5 and 12; red arrowheads point at xbra expression in the involuted mesoderm. Graphs show percentages of embryos with normal vs. abnormal expression of each marker; N = 32–40 embryos per Control or fgfr4 CRISPR; **p < 0.001, ***p < 0.0001.

Sempou et al. FGFR4 in LR Patterning

to affect LR patterning via SM patterning, most of them via the ciliogenesis gene foxj1 (Caron et al., 2012; Walentek et al., 2012; Griffin et al., 2018). In addition, knockdown of the global mesodermal determinant Brachyury/xbra results in LR defects both in frogs and mice (King et al., 1998; Kitaguchi et al., 2002). However, little is known about the signals that determine the fate of the PSM portion of the GRP during gastrulation, and while SM specification has been previously connected to candidate heterotaxy genes (Griffin et al., 2018), it is unclear whether PSM specification is similarly relevant. The PSM GRP is part of the greater paraxial mesoderm and thus expresses myogenic markers during gastrulation (Shook et al., 2004; Schweickert et al., 2010). After gastrulation, the layer of PSM exposed to the gastrocoel becomes distinct from the more superficial PSM by additional patterning through factors (e.g., coco, xnr1, and gdf3) that facilitate its role as LRO tissue and enable the onset of asymmetric gene expression. At that stage, the PSM of the GRP still maintains the expression of myogenic factors like myoD (Schweickert et al., 2010). Given the key role of the PSM in the frog GRP, it is not surprising that paraxial mesoderm specification affects LR patterning. In mammals, the exact lineage of node cells and thus the contribution of paraxial mesoderm to the LRO remains to be determined. In mice, Fgfr4 expression is detectable in paraxial myogenic mesoderm during node stages (Stark et al., 1991), and it would be interesting to examine whether its transcripts are present in the node region before LR cues become upregulated. Given that there are no reported LR phenotypes upon knockdown of the few known genes required for muscle development (Rudnicki et al., 1993; Pownall et al., 2002), it seems unlikely that the myogenic properties of paraxial mesoderm per se affect LRO function. It is rather likely that fgfr4 controls an array of paraxial mesodermal genes during gastrulation, and that one or more of these genes are key to specify the GRP for LR cue expression.
FGF ligands FGF8 and FGF4 are required at several steps of LR development, which include LRO morphogenesis, ciliogenesis, and asymmetric gene expression at the LPM (Boettger et al., 1999; Meyers and Martin, 1999; Albertson and Yelick, 2005; Yamauchi et al., 2009). In addition, FGFR1 has been identified as essential for ciliogenesis once the LRO is shaped (Neugebauer et al., 2009). The role of FGFR4 appears distinctly different than that of FGFR1, since it acts to specify LRO tissue during gastrulation and is not expressed in the established LRO. We also observe fewer and sometimes shorter cilia in GRPs of fgfr4 CRISPR embryos, but this effect is likely secondary to the patterning defect.

A long-standing connection exists between FGF signaling and gastrulation, and mesodermal patterning and morphogenesis in particular. Expression of dominant negative constructs for FGFR1 and FGFR4 effectively perturbs mesoderm induction by abolishing xbra expression (Amaya et al., 1991, 1993; Isaacs et al., 1994; Hardcastle et al., 2000). Moreover, depletion of ligand FGF4 (eFGF) strikingly resembles fgfr4 knockdown by inhibiting myoD expression (Fisher et al., 2002) in the early mesoderm, suggesting that a fgfr4/fgfr4 interaction may convey paraxial mesodermal specification during gastrulation.

Altogether our study establishes a link between FGFR4 and induction of paraxial mesoderm during gastrulation, which impinges on the specification of the pre-somatic GRP and its function as a LRO. These results help to construct the complex puzzle of FGF ligands and receptors that contribute to mesodermal and LR patterning in the early embryo.

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SUPPLEMENTARY MATERIAL
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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