



Abnormalities in Voltage-gated Calcium Channels in Patients with Autism Spectrum Disorder: A Systematic Review

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Abstract. Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder with delayed social communication and behavior skills. Scientists have postulated various theories to understand the different mechanisms involved in the pathogenesis of ASD with consistent findings of mutations in genes encoding Voltage-gated Calcium channels (VGCCs) in ASD patients. We have systematically reviewed and analyzed various mutations in VGCC genes and their association with ASD. We did a literature search in PubMed, Cochrane Database of Systematic Reviews, NIH database for clinical trials, and Google Scholar. We used keywords “Autism Spectrum Disorder”, “Autistic Disorder”, “Calcium channels”, and the filter terms etiology and genetics. We included all free full-text articles in the English language that were published from 2005 to 2020. This review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocol (PRISMA-P) 2009 guidelines. We selected 14 studies for this review after applying exclusion criteria and performing Quality Assessment (QA). All of our studies show a significant association between ASD and mutations in VGCC genes. These findings support further testing of the readily available Calcium channel blockers (CCBs), with known side effects and safety profile, and understand if CCBs can be repurposed for treating or alleviating the symptoms of ASD.

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1. Introduction:

One in 160 children worldwide is diagnosed with ASD, with the actual figures significantly higher than estimated (WHO, 2019). In the United States, the prevalence of ASD in children eight years of age was 6.7 (one in 150 children) between 2000 and 2002, which increased more than twice in 2014 to 16.8 per 1,000 (one in 59 children) (Maenner et al. 2020). The spiking rate of ASD has posed several problems for patients and their families, including decreased quality of life, co-morbid mental health problems, emotional turmoil, marital discordance, and an overwhelming financial burden for families and healthcare systems. Regarding finances, the annual expenses (including medical, non-medical, loss of productivity) estimated for 2015 was \$268 billion, which was equal to the cost estimated for diabetes and Attention deficit hyperactivity disorder (ADHD). If the prevalence of ASD continues to rise at the same pace, its economic burden will surpass Diabetes and ADHD by 2025 (Leigh et al. 2015).

ASD is a neurodevelopmental disorder that involves varying degrees of challenges in social interaction,

communication, and restricted/repetitive activities. A wide range of co-morbid medical conditions associated with ASD includes gastrointestinal abnormalities, immune dysfunction, ADHD, developmental delay, epilepsy, depression, and anxiety (WHO. 2019, Nguyen et al. 2018). The risk of ASD is 20-80 times higher in siblings of affected individuals, which points to its high heritability and has led to a robust search for genes and biochemical pathways involved in ASD (Nguyen et al. 2018, Li et al. 2015). Many genome-wide association studies (GWAS) and whole exome sequencing studies have repeatedly recognized the association between ASD and mutations in genes encoding VGCC and calcium signaling pathways (Ebert et al. 2013, Liao et al. 2020, Splawski et al. 2006, Pinggera et al. 2017). These findings strongly reinforce calcium channel genes as a vital gene involved in the pathogenesis of ASD (Liao et al. 2020).

VGCCs are transmembrane proteins vital for key physiological processes implicated in the electrical excitability of cells, which contribute to the synaptic plasticity, secretion of hormones and neurotransmitters, contraction of muscles, regulation of pacemaker activity,

and expression of genes (Catterall, 2011). Scientists have divided VGCCs into high voltage-activated channels (HVA) and low voltage-activated channels (LVA) based on their threshold activation. HVA includes L-, N-, P/Q- and R-types channels, whereas LVA consists of T-type channels (Catterall, 2011). The central pore-forming unit is the $\alpha 1$ subunit, which has three auxiliary subunits; a disulfide-linked $\alpha 2\delta$ (Cav $\alpha 2\delta$) subunit, an intracellular β subunit (Cav β), and a transmembrane γ subunit (Cav γ) (Daghni et al. 2018). Figure 1 shows the structure of VGCCs. Although the $\alpha 1$ subunits establish the principal pharmacological properties, voltage dependence, and kinetics of VGCCs, the auxiliary subunits can also alter the function as they control the trafficking and membrane targeting of VGCCs (Catterall, 2011).

The L-type channels comprise CaV1.1–CaV1.4 channels encoded by genes CACNA1S, C, D, and F, respectively. The P/Q-, N-, and R-type channels include CaV2.1–CaV2.3 encoded by genes CACNA1A, B, and E, respectively. The T-type channels consist of the CaV3.1–CaV3.3 channels, encoded by genes CACNA1G, H, and I, respectively (Liao et al. 2020, Heyes et al. 2015). Likewise, four types of β subunits and $\alpha 2\delta$ subunits are identified and are encoded by genes CACNB1–4 and CACNA2D1–4, respectively (Catterall, 2011, Heyes et al. 2015). Calcium channel inactivation is a crucial mechanism by which cells can tightly control intracellular calcium levels and, therefore, excitable cells' activity (Catterall, 2011). Mutations in VGCC genes are associated with various neuropsychiatric disorders, including ASD (Pinggera et al. 2017, Andrade et al. 2019). These studies confer a need to thoroughly explore the genes implicated repeatedly in ASD patients, which can subsequently help us understand the pathogenesis of ASD. It will also help explore novel therapeutic interventions that can help treat or alleviate the symptoms in ASD patients.

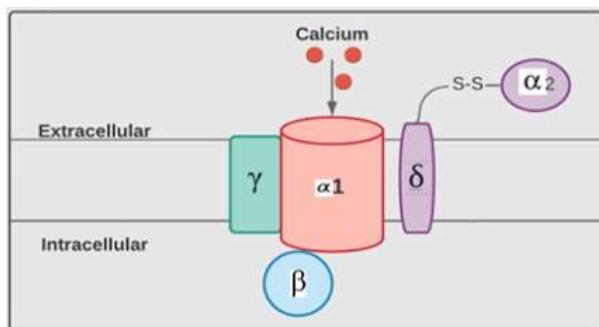


Figure 1. Structure of Voltage-gated Calcium channels. The central $\alpha 1$ subunit is a transmembrane protein through which calcium ions pass. The auxiliary subunits include a disulfide-linked $\alpha 2\delta$ subunit, an intracellular β subunit, and a transmembrane γ subunit

This review aims to analyze and summarize the existing evidence regarding abnormalities in VGCCs in patients with ASD.

2. Method:

This study's protocol follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocol (PRISMA-P) 2009 guidelines (Moher et al. 2015).

We conducted an online literature search in PubMed, Cochrane Database of Systematic Reviews, NIH database for clinical trials, and Google Scholar. We included studies that assessed abnormalities in VGCCs and their role in the pathogenesis of ASD. The search was conducted from November 10th, 2020 to January 17th, 2021. We included all free full-text articles in the English language that were published from 2005 to 2020 to get the most recent studies. We did not place any restrictions on the location of the studies or the age of the participants. Animal studies were excluded.

The search was conducted in PubMed using the MeSH and Regular keywords, "Autism Spectrum Disorder" OR "Autistic Disorder" and using the filter terms etiology and genetics. We also searched for MeSH and Regular keywords, "Calcium channels" and used filter terms genetics. These keywords were used in various combinations using Boolean operators like "OR" and "AND". We searched Google Scholar and Cochrane Database of Systematic Reviews using keywords, Autism Spectrum Disorder OR Autistic Disorder AND Voltage-gated Calcium channels. We also examined the reference list of selected studies to understand if any research was used as a reference in multiple articles and substantially impacted the searched literature.

The data were extracted using a standardized form. One author extracted the data, and the other author verified the accuracy of the data. Any questions or concerns were discussed between the authors until we reached a mutual consensus. Many articles discussing the role of VGCCs in ASD have included the role of calcium signaling pathways in their studies. As this topic was out of our study's scope, we did not include the specific details about it in our review. Data extracted consists of the first author's name, publication year, type of study, sample size, VGCC genes, mutations in genes, and key findings.

The QA screening was done by two reviewers independently using the QA tools to assess the risk of bias. We used the CARE checklist for case report/series and Newcastle-Ottawa Scale for observational studies (Gagnier et al. 2013, Lo et al. 2014). We only included the studies that satisfied QA requirements in this systematic review.

3. Result:

The initial search in PubMed, combining the MeSH and Regular keywords, identified a total of 109,541 studies. After applying the eligibility criteria, we found a

total of 10,592 results from all databases. However, we could only retrieve the first 1000 articles from Google Scholar. All the results were managed using the Mendeley Reference Manager, and after removing the 87 duplicate studies, 10,505 studies remained for further evaluation.

Two authors evaluated the titles and removed 10,421 irrelevant articles.

Finally, 84 studies were found relevant and selected for full-text review by two independent reviewers. We excluded 57 articles after mutual discussion and 13 articles that did not pass the QA. After QA, we finalized 14 papers to be eligible for this systematic review.

Figure 2 shows the flow chart with details about study selection.

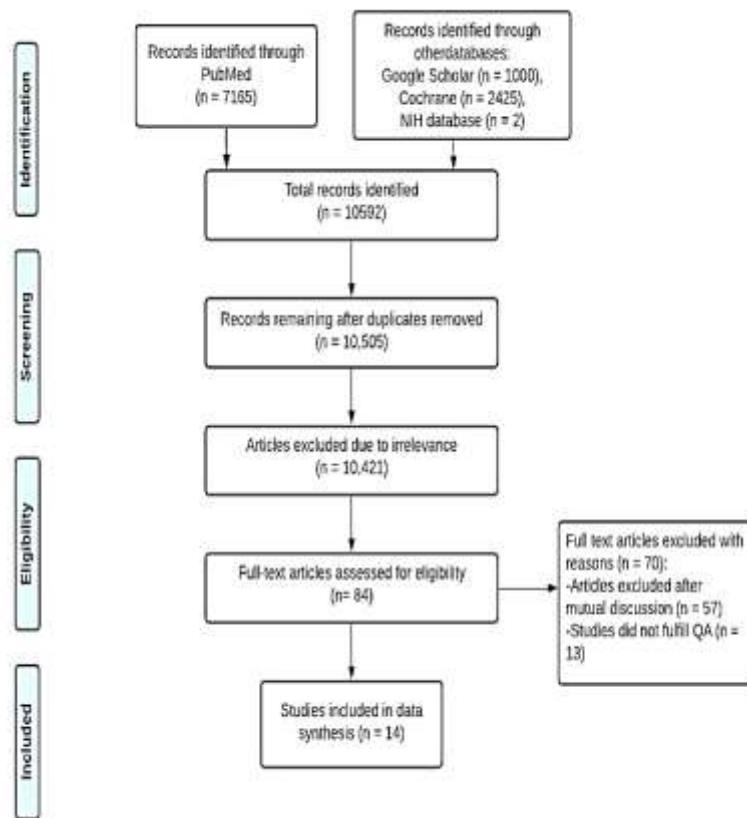


Figure 2. Study flow diagram

The selected 14 articles included 12 observational studies, one case report, and one case series. We assessed all the studies by individual QA tools. We used the Newcastle-Ottawa Scale for observational studies and the CARE checklist for case reports/series.

All studies fulfilled the quality check. We have summarized the key findings from various studies about VGCCs and their association with ASD in Table 1.

Table 1. Key information about studies that show mutations in VGCCs and their association with ASD and other neuropsychiatric disorders

Study	VGCC, associated Gene	Sample	Abnormality	Key findings
Li et al., 2015	CaV2.1, CACNA1A	1659 ASD patients	SNPs	Alleles of rs7249246 and rs12609735 in CACNA1A were found in ASD patients.

Table 1. Key information about studies that show mutations in VGCCs and their association with ASD and other neuropsychiatric disorders

Study	VGCC, associated Gene	Sample	Abnormality	Key findings
Splawski et al., 2006	CaV3.2, CACNA1H	461 ASD patients, 480 controls	missense mutation – loss of function	Low incidence of four mutations, R212C, R902W, W962C, and A1874V, were found that affected voltage-dependent activation in ASD patients
Pinggera et al., 2017	CaV1.3, CACNA1D	One ASD patient	Missense mutation, gain-of-function	V401L mutation in CACNA1D that caused gating changes in VGCCs in an ASD patient
Li et al., 2015	CaV1.2, CACNA1C	553 trios	SNPs	Alleles of rs1006737 and rs4765905 were found in patients with ASD
Pinggera et al., 2015	CaV1.3, CACNA1D	2303 trios	Missense mutation, gain-of-function	Two de novo mutations, p. A749G and p. G407R, were found to be associated with ASD
Cross-Disorder group, 2013	CaV1.2, CACNA1C; β subunit, CACNB2	33,332 cases and 27,888 controls	SNPs	SNPs in CACNA1C (rs1024582) and CACNB2 (rs2799573) were found to be related to ASD and four other psychiatric disorders
Damaj et al., 2015	CaV2.1, CACNA1A	16 individuals	Loss-of-function mutation	Mutations in CACNA1A were found to be associated with ASD
Strom et al., 2009	CaV3.1, CACNA1G	284 MO (male only) trios	SNPs	Alleles of rs757415 and rs12603112 were significantly over transmitted within CACNA1G in ASD patients
Lu et al., 2012	CaV3.3, CACNA1I; CaV3.1, CACNA1G; CaV1.2, CACNA1C	2781 trios	SNPs	Rs10848653 in CACNA1C, rs198538, and rs198545 in CACNA1G, rs5750860 in CACNA1I, were found to be related to ASD

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Study	VGCC, associated Gene	Sample	Abnormality	Key findings
Breitenkamp et al., 2014	β subunit, CACNB2	155 ASD patients, 259 controls	missense mutations – gain of function	Mutations in G167S, S197F, and F240L that altered the current kinetics of CaV1.2 subunit in ASD patients
Lencz and Malhotra, 2015	CaV1.2, CACNA1C; β subunit, CACNB2; CaV3.3, CACNA1I	1500 cases and 66,000 controls	SNPs	iGenes identified through GWAS
Iossifov et al., 2012	$\alpha 2\delta$ subunit, CACNA2D3	343 families	De novo variant	Mutations in CACNA2D3 that disrupted the protein function were found through exome sequencing
Girirajan et al., 2013.	$\alpha 2\delta$ subunit, CACNA2D3	2588 ASD patients, 580 controls	CNV	Deletions in CACNA2D3 were found in ASD patients
Prasad et al., 2012	$\alpha 2\delta$ subunit, CACNA2D4	696 ASD patients, 1000 controls	CNV	Mutations in CACNA2D4 were reported in patients with ASD

ASD: Autism Spectrum Disorder; VGCCs: Voltage-gated Calcium channels; SNP: Single Nucleotide Polymorphisms; GWAS: Genome-wide Association Studies; CNV: Copy Number Variation.

4. Discussion:

We found 14 studies that identify specific mutations in VGCC genes in ASD patients. Two studies identified CACNA1A, CACNA1D, CACNA1G, and CACNA1I, four studies identified CACNA1C, one study identified CACNA1H, and three studies identified genes encoding β subunit and $\alpha 2\delta$ subunit of the VGCC as the candidate genes for ASD. Different techniques have been utilized by genomic studies to determine the genes implicated in neuropsychiatric disorders, including ASD (Demkow et al. 2017). GWAS has been used repeatedly to identify genetic variations, including single nucleotide polymorphisms (SNPs), copy number variations (CNVs), de novo variations, and large chromosomal rearrangement (Li et al. 2015, Cross Disorder Group 2013, Damaj et al. 2015, Strom et al. 2009, Lu et al. 2012, Breitenkamp et al. 2014, Lencz et al. 2015, Girirajan et al. 2013, Prasad et al.

2012). Another popular method used is whole exome and genome sequencing that analyzes the coding regions of thousands of genes simultaneously (Pinggera et al. 2015, Iossifov et al. 2012). All the studies have consistently stressed the polygenicity in the ASD population, with a vast number of coding and non-coding loci being polymorphic (Daghsni et al. 2018, Heyes et al. 2015). SNPs represent the common alleles, each with minor effects. In contrast, CNVs and deleterious mutations represent rare alleles, with comparatively increased risk, and together both alleles confer the genetic risk of complex phenotype in ASD (Heyes et al. 2015, Lencz et al. 2015, Iossifov et al. 2012, Girirajan et al. 2013).

Pinggera et al. discussed a case report that identified de novo missense mutation (V401L) in the CACNA1D gene in an ASD patient, which decreased channel

inactivation and increased calcium influx through the channel, resulted in a gain-of-function phenotype (Pinggera et al. 2017). Moreover, the mutant channels sustained complete sensitivity towards isradipine, a Calcium channel blocker (CCB). This finding suggested that CCB might improve psychiatric symptoms in patients with gain-of-function mutations in CACNA1D. The presence of Cav1.3 in the amygdala and striatum, which are the parts of the brain implicated in ASD in many studies, further supports these findings (Pinggera et al. 2017). The major limitation of this study is the small sample size. Pinggera et al. conducted another experiment with a larger sample size of 2303 trios. They concluded the presence of two de novo mutations, p.A749G and p.G407R in the CACNA1D gene, found only in the probands and were absent in their unaffected parents or siblings (Pinggera et al. 2015). Both mutations had a gain-of-function phenotype due to the prevention of voltage-dependent inactivation of CaV1.3, resulting in increased calcium influx. Li et al. and Damaj et al. identified CACNA1A as a candidate gene for ASD in studies with a sample size of 1659 ASD patients and 16 ASD patients, respectively (Li et al. 2015, Damaj et al. 2015). Li et al. conducted family-based association research within the Chinese Han population and found SNPs in rs1006737 and rs4765905 were associated with ASD (Li et al. 2015). However, they could not reach statistical significance after Bonferroni correction, which is a limitation in this study. Damaj et al. found loss-of-function mutations in the CACNA1A genes in ASD patients. On sequencing, they found one CACNA1A gene deletion, two CACNA1A point mutations, and one new frameshift variant, and a new splice-site variant. These mutations were also associated with other neurobehavioral disorders like episodic ataxia and epilepsy in their subset of the patient population (Damaj et al. 2015). This study also has a small sample size.

Splawski et al. conducted a case-control study with 461 cases and 480 controls (all Caucasians) and found four missense loss-of-function mutations (R212C, R902W, W962C, and A1874V) in the CACNA1H gene in six of 461 ASD cases. These mutations were found in the critical region of the protein and produced lesser currents that decreased current conductance, causing decreased channel activity (Splawski et al., 2006). Some discrepancies found in the study include the low incidence of mutation, absence of mutation in an affected child, and the same mutations in unaffected individuals, which concludes that these mutations might be a minor contributor to ASD patients as many studies suggest polygenic inheritance of ASD. Breitenkamp et al. recognized three rare missense mutations, G167S, S197F, and F240L, in CACNB2 in a study involving 155 ASD cases and 259 controls, all Caucasians (Breitenkamp et al., 2014). These mutations were found in three out of 155 ASD patients and were absent in the control group. The CaV β -subunit controls the

activity and regulation of L-type calcium channels. These mutations altered the current kinetics by slowing down the time-dependent inactivation and increasing the sensitivity of voltage-dependent inactivation. These changes disrupted the proper functioning and homeostasis of the calcium channels in ASD patients. Li et al. analyzed 553 trios of ASD families, investigating the association between ASD and SNP in CACNA1C (Li et al., 2015). They found preferential transmission of the G allele of rs1006737 and G allele of rs4765905 from parents to affected ASD offspring. Lu et al. performed a study on 2,781 trios and found four SNPs in three VGCC genes in ASD patients (Lu et al., 2012). They found rs10848653 located in CACNA1C, rs198538, and rs198545, located in CACNA1G, and rs5750860 located in the CACNA1I gene. The SNPs exceeded the corrected level of significance after the Bonferroni correction, further supporting the study's findings. Strom et al. analyzed 284 male-only trios of ASD and identified alleles of rs757415 and rs12603112 being significantly over-transmitted within CACNA1G in ASD patients (Strom et al., 2009). A few limitations in this study include the findings of a follow-up bias test that did not yield the former allele, limiting original research to only male patients, and small sample size. Cross-Disorder Group performed the most extensive genome-wide analysis of five psychiatric disorders, including ASD, depression, ADHD, bipolar disorder, and schizophrenia (Cross-Disorder Group, 2013). They identified SNPs within CACNA1C and CACNB2 in all five conditions. The SNPs within these two genes exceeded the cutoff for genome-wide significance. Some limitations in this study include chances of misdiagnosis that could have over or underestimated genetic overlap, sample restricted to European ancestry, and comparison based on a selection of models that had a better fit than any alternate model.

The mutations in the $\alpha 2\delta$ subunit were found in three studies. Iossifov et al. conducted their study on 343 families with only one affected child and one or more normal siblings. They concluded that gene disrupting splice site mutations in CACNA2D3 were twice as frequent in ASD patients as their unaffected siblings. Their study found no evidence of missense mutations in autistic individuals (Iossifov et al., 2012). The results were validated by a PCR-amplified DNA test from family members, which further support their findings. In another study with a larger sample size, Girirajan et al. identified recurrent CNVs in ASD patients, specifically deletions in the CACNA2D3 gene (Girirajan et al., 2013). They further found a decline in the non-verbal IQ in ASD patients with an increased size of deletions, while no effect on non-verbal IQ was observed with the increased size of duplications. Prasad et al. used both single-nucleotide polymorphism (SNP) arrays and comparative genomic hybridization (CGH) arrays to detect CNVs in their sample population (Prasad et al., 2012). They noticed many CNVs in the ASD patients, including loss of the CACNA2D4

gene. Using CGH, they were able to detect smaller CNVs that are usually missed by SNP microarrays.

All the studies analyzed supports the role of VGCCs genes in ASD pathogenesis. However, the low penetrance of genes in many studies suggests that the many mutations represent rare polymorphism. Therefore, more extensive and ethnically diverse samples are needed to understand the overall effect of these mutations. Another discrepancy in data about the properties of gain or loss-of-function of VGCCs is hard to comprehend with currently available data. Since gene transcription requires a certain amount of calcium concentration for regulated function, we assume that changes in either direction can give rise to developmental abnormalities. These findings altogether pose a new question: to explore if specific mutations in VGCC genes are responsible for causing a milder form of ASD, compared to others that might cause a severe form of ASD. Although many genes other than VGCC genes have been linked to ASD pathogenesis, VGCC genes can have future therapeutic implications due to readily available CCBs (Andrade et al. 2019, Harrison et al. 2019). CCBs are licensed drugs with known safety and side effects profile, making them a cost-effective, safe, and fast track choice for treating neuropsychiatric disorders. Many studies have identified mutations in VGCC genes in other neuropsychiatric disorders like depression, ADHD, Bipolar disorder, schizophrenia, epilepsy, and anxiety disorders (Pinggera et al. 2017, Heyes et al. 2015, Andrade et al. 2019, Cross-Disorder Group 2013, Damaj et al. 2015). However, available studies on testing CCBs in neuropsychiatric disorders are limited with mixed results. Moreover, they do not provide a clear consensus on the decisive role of CCBs in treating neuropsychiatric disorders (Hayes et al. 2019, Cipriani et al. 2016, Mallinger et al. 2008). Given the consistent involvement of VGCC genes in ASD patients, we suggest CCBs with selectivity for brain-enriched isoforms as a potential therapeutic option in treating or alleviating the symptoms of ASD.

4. Limitation:

We used studies with free full access and written in English language only from 2005 to 2020. Thus, relevant articles of closed access, written in other languages and published before 2005, may have been skipped.

5. Conclusion:

This systematic review analyzes and reviews the abnormalities in VGCCs in patients with ASD. Our findings consistently indicate a strong association between ASD and mutations in VGCCs, with genes encoding channels on $\alpha 1$ subunit, $\alpha 2\delta$ subunit, and β subunit. Genomic studies, including GWAS and whole-exome sequencing, identified multiple SNP, CNVs, de novo mutations, and gene-disrupting mutations (nonsense, splice site, and frameshifts) in VGCC genes in ASD patients. Integration of this data on individual genetic variations

with other disruptive genes, and their overall interaction with the environment, can help understand the compounded effect of these variations on the pathogenesis of ASD. Future researchers can also explore if specific mutations in VGCC genes are responsible for causing a milder form of ASD than others that might cause a severe form of ASD. Exploring this question can provide helpful information on the overall impact of VGCC mutations in ASD pathogenesis. Moreover, based on our findings, we support the idea to critically test CCBs with selectivity for brain-enriched isoforms in ASD patients as they are readily available and used worldwide.

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Statement of interest:

All authors of this paper declare no conflict of interest related to this manuscript's content.

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