<table>
<thead>
<tr>
<th>Title</th>
<th>Pentameric viral ion channels : from structure to function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Surya, Wahyu; Li, Yan; Torres, Jaume</td>
</tr>
<tr>
<td>Citation</td>
<td>Surya, W., Li, Y., &amp; Torres, J. (2014). Pentameric viral ion channels : from structure to function. Journal of receptor, ligand and channel research, 2015(8), 9-18.</td>
</tr>
<tr>
<td>Date</td>
<td>2015</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10220/25365">http://hdl.handle.net/10220/25365</a></td>
</tr>
</tbody>
</table>

© 2015 Surya et al. This work is published by Dove Medical Press Limited, and licensed under Creative Commons Attribution - Non Commercial (unported, v3.0) License. The full terms of the License are available at http://creativecommons.org/licenses/by-nc/3.0/. Non-commercial uses of the work are permitted without any further permission from Dove Medical Press Limited, provided the work is properly attributed. Permissions beyond the scope of the License are administered by Dove Medical Press Limited. Information on how to request permission may be found at: http://www.dovepress.com/permissions.php
Pentameric viral ion channels: from structure to function

Wahyu Surya
Yan Li
Jaume Torres

School of Biological Sciences, Nanyang Technological University, Singapore

Abstract: A family of small polypeptides in many virus types associate to form oligomers and have channel activity. These proteins have been referred to as viroporins or virochannels and are increasingly recognized as important virulence factors and potential drug targets. In this review, we focus on two of the viroporins that have been studied in more detail from a structural and functional point of view. One is the 76-residue envelope (E) protein found in coronaviruses (CoVs) that causes the severe acute respiratory syndrome (SARS). The other is the 65-residue small hydrophobic (SH) protein found in a paramyxovirus, the respiratory syncytial virus (RSV). RSV SH and SARS-CoV E proteins are short polypeptides with a single transmembrane domain. In both cases, the presence of the viroporin has a protective effect on cells, preventing early apoptosis, but it leads to increased virulence in infected animal models. Both viroporins form homopentameric oligomers that show channel activity with no or low selectivity. The role of channel activity is still unclear, but associations have been made to facilitation of the egress of the virus by modification of the secretory pathway, and contributions to inflammation. SARS-CoV E protein has a cytoplasmically oriented C-terminus and a luminal N-terminus, whereas the opposite orientation is found in RSV SH protein. Despite this opposite topology, nuclear magnetic resonance (NMR)-based structural models of these two channels show a similar champagne flute shape, with the wider opening facing the cytoplasmic side. Good channel inhibitors are lacking, but those found seem to have a preference for the narrow end of the channel. Availability of good inhibitors will help reveal the specific role of these channels in the life cycle of these viruses.

Keywords: respiratory syncytial virus, severe acute respiratory syndrome, small hydrophobic protein, envelope protein, channel structure

Viroporins

The term “viroporins” was proposed about 20 years ago after the observation of enhanced membrane permeability in virus-infected cells. Viroporins are a class of small proteins (~60–120 amino acids long) with one or two hydrophobic transmembrane (TM) domains that oligomerize to produce ion channels (hydrophilic pores) at the membranes of the host cell. In addition to the best studied case, the 97-residue M2 protein in influenza A,4–6 many viruses encode viroporins, for example, p7 in the hepatitis C virus (HCV),7 Vpu in human immunodeficiency virus type 1,5 2B in picornavirus,6 6K in alphavirus,8 p10 in avian reovirus,11 p7 in bovine viral diarrhea virus,12 Kcv in paramecium bursaria chlorella virus 1,13 and the list of members of this class of viral proteins keeps growing.7–20 The two that will be the focus of our review, the small hydrophobic (SH) protein in the respiratory syncytial virus (RSV),18 and envelope (E)
proteins in coronaviruses (CoVs),\textsuperscript{21,22} are among the very few that have been studied structurally in some detail.

**Envelope proteins in CoVs**

CoVs are enveloped viruses organized into three groups:\textsuperscript{23} group 1 (\(\alpha\)-CoVs) includes the porcine transmissible gastroenteritis virus (TGEV) and human CoV 229E, group 2 (\(\beta\)-CoVs) includes the murine hepatitis virus (MHV), severe acute respiratory syndrome (SARS)-CoV and Middle East respiratory syndrome (MERS)-CoV, and group 3 (\(\gamma\)-CoV) includes the avian infectious bronchitis virus (IBV). Representative sequences of these groups are shown in Figure 1A. Of particular medical interest are the SARS-CoV, which produced a near pandemic in 2003, and the recently emerged MERS-CoV, with 837 confirmed cases and at least 291 deaths (http://www.who.int/csr/don/2014_07_23_mers/en/).

CoV E proteins are short (76–109 amino acids) structural membrane proteins present at low concentration in the CoV virion,\textsuperscript{20,24–26} but found abundantly in internal membranes of infected cells, from ER to Golgi.\textsuperscript{20,27–29} For example, MHV E and SARS-CoV E are found localized at the ER-Golgi intermediate compartment, where CoV virions are assembled and bud into the lumen.\textsuperscript{30,31}

E proteins have a predicted \(\alpha\)-helical TM domain (Figure 1B), with two or three cysteine residues at the juxtamembrane region. Determination of the topology of E proteins has been confused by the fact that epitope tags can affect the topology of these small proteins.\textsuperscript{32–34} However, a recent study\textsuperscript{31} of untagged SARS-CoV E protein showed N\textsubscript{exo} C\textsubscript{cyto} topology in infected cells, that is, a cytoplasmic C-terminal domain and a lumenal N-terminus, and this topology is also likely in other E proteins.\textsuperscript{26,29,31,32}

Deletion of E protein (\(\Delta E\)) is deleterious or otherwise attenuating to the virus.\textsuperscript{35,36} SARS-CoV \(\Delta E\), for example, grows to a lower titer and shows aberrantly shaped virions.\textsuperscript{37} In vivo, this virus is highly attenuated,\textsuperscript{38} which has led to the development of a live vaccine.\textsuperscript{39} This route seems to be highly promising based on mice trial results\textsuperscript{40} and has also been suggested as a way to curb the spread of MERS-CoV.\textsuperscript{41}

Infected cells by SARS-CoV \(\Delta E\) underwent apoptosis more rapidly and to greater extent, showed upregulation of stress response genes, downregulation of inflammation genes,\textsuperscript{42} and decreased nuclear factor kappa-light-chain-enhancer of activated B cells-mediated inflammation response in infected cells and mice.\textsuperscript{43}

**Structure of the envelope protein in SARS-CoV**

The SARS-CoV E protein structure was initially investigated using synthetic peptides corresponding to its predicted TM domain and molecular dynamics simulations.\textsuperscript{44} A pentameric model was proposed that was later confirmed using infrared linear dichroism data from \textsuperscript{13}C=\textsuperscript{18}O isotopically labeled synthetic peptides incorporated in lipid bilayers.\textsuperscript{45,46} While cross-linking studies with full-length tagged SARS-CoV E-protein indicated undefined oligomers,\textsuperscript{20,21} the pentameric

![Figure 1](image_url) 

**Figure 1** Sequence alignment and secondary structure prediction of representative CoV envelope proteins. 
**Notes:** (A) Alignment of E proteins in representatives from \(\alpha\)-CoV (TGEV), \(\alpha\)-coronavirus (MHV and SARS-CoV), and \(\gamma\)-coronavirus (IBV). A conserved polar residue and Proline are indicated by an arrow and star, respectively; (B) secondary structure and TM domain prediction. 
**Abbreviations:** CoV, coronavirus; TGEV, the porcine transmissible gastroenteritis virus; MHV, murine hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; IBV, infectious bronchitis virus; TM, transmembrane.
nature of the oligomer has been confirmed using analytical ultracentrifugation of the full-length polypeptide. Current data suggest that disulfide bonds, or the presence of juxtamembrane cysteines, are not required for oligomerization for TGEV E, IBV E, MVH E, or SARS E. A more detailed study of the TM structure of SARS-CoV E was obtained from solution NMR of a synthetic isotopically labeled E (8–38) in dodecylphosphocholine (DPC) detergent micelles. A model of the pentamer was derived from intermonomeric Nuclear Overhauser effects and paramagnetic relaxation enhancement data, confirming the TM helix orientations reported previously. Although the resolution of the details is still low, these data produced a consistent picture of the channel geometry, where Val25 is located at the narrowest point of the lumen (Figure 2A).

Interestingly, the predicted β-structure region, or hairpin, centered on a totally conserved Pro residue (Figure 1), formed 100% β structure when studied as a short peptide, but was found to be mostly α-helical in the context of a longer construct E (8–65; Figure 2B) and also full-length.

![Figure 2](image-url) NMR-based models of SARS-CoV E proteins.

Notes: (A) Pore radius in the pentameric bundle formed by the TM domain of SARS-CoV E, ETM (8–38), showing the residues lining the hydrophilic pore; (B) solution NMR structure of E (8–65) in SDS and α-helical conformation near the conserved Pro residue at the C-terminal tail; (C) side and cytoplasmic view of a pentameric bundle of SARS-CoV E derived from A and B. Each monomer is colored differently and extramembrane intermonomeric contacts are indicated. (A) Adapted from Pervushin K, Tan E, Parthasarathy K, et al. Structure and inhibition of the SARS coronavirus envelope protein ion channel. PLoS Path. 2009;5(7):e1000511. (B) This research was originally published in The Journal of Biological Chemistry. Li Y, Suryawanshi, Claudine S, Torres J. Structure of a conserved golgi complex-targeting signal in coronavirus envelope proteins. J Biol Chem. 2014;289(18):12535–12549. © the American Society for Biochemistry and Molecular Biology.

**Abbreviations:** NMR, nuclear magnetic resonance; SARS-CoV, severe acute respiratory syndrome coronavirus; TM, transmembrane; SDS, sodium dodecyl sulphate.
SARS-CoV E.\textsuperscript{48} This \( \alpha \)-helical conformation is consistent with circular dichroism and Fourier transform infrared spectroscopy data.\textsuperscript{47} Overall, this discrepancy may point to the existence of a frustrated structure poised for conformational change in this part of the molecule. These data have resulted in a mostly \( \alpha \)-helical model for SARS-CoV E that is reminiscent of that of HCV p7 (Figure 2C).\textsuperscript{50}

**Channel activity of SARS-CoV E and its pathological role**

Channel activity with very mild cation selectivity has been observed in black lipid membrane (BLM) systems using E protein synthetic peptides encompassing their predicted TM region.\textsuperscript{51,52} Later studies have shown minor changes in selectivity induced by lipid charge\textsuperscript{53} and pH,\textsuperscript{54} and the N-terminal Glu residues in SARS-CoV E have been suggested to confer some selectivity.\textsuperscript{54} A truncated form of SARS-CoV E (8–65) in DPhPC (diphytanoylphosphatidylcholine) showed a conductance of 0.39±0.02 nS.\textsuperscript{48} For comparison, synthetic full-length SARS-CoV E and E (7–38) produced single channel conductances of 0.19±0.06 nS and 0.18±0.12 nS in 1 M NaCl,\textsuperscript{53} respectively. The lower conductance observed in synthetic samples may be due to extraneous modifications or impurities resulting from exposure to harsh chemicals.

Despite initial patch clamp data reports showing cation selective channel activity,\textsuperscript{19} SARS-CoV E protein could not be detected at the plasma membrane of transfected cells\textsuperscript{53} or in frog oocytes.\textsuperscript{55} In the latter system, co-expression of SARS-CoV E with human epithelial sodium transporter in Xenopus oocyte decreased amiloride-sensitive current through activation of protein kinase C and subsequent decrease in epithelial sodium transporter surface levels.\textsuperscript{55} A similar direct or indirect inhibitory effect on other endogenous channels was observed by patch clamp in transfected cells.\textsuperscript{51}

Early indications showed that channel activity of E proteins may be important for CoVs. For example, titer, plaque size, and hexamethylene amiloride (HMA) sensitivity was reduced when parts of the TM were rotated by insertion of Ala residues in various positions.\textsuperscript{56} Synthetic peptides of SARS-CoV E carrying several mutations have contributed to identify residues that abolish ion channel activity, that is, Val25 and Asn15.\textsuperscript{57} The latter is in a conserved position in the TM domain of all E proteins, were a polar residue is always found (Figure 1A). The side chains of these two residues face the luminal side and monomer–monomer interface, respectively.\textsuperscript{58}

Consistently, SARS-CoV carrying E protein with channel-inactivating N15A and V25F mutations was attenuated in vivo and led to the appearance of compensatory mutations that rapidly outcompeted the inactive mutants.\textsuperscript{59} Mutation N15A was compensated by A15D, whereas pentamer-disruptive mutation V25F was compensated at the neighbor \( \alpha \)-helix in the pentameric channel that faces residue 25.\textsuperscript{19,63} This is consistent with the primary destabilization of the pentameric oligomer by V25F. Mutation N15A, in contrast, does not destabilize the oligomer (results unpublished); therefore, inhibition may be related to a more fundamental aspect of channel activity and interaction with the ions.

These revertant mutants restored ion channel activity and mortality comparable to wild-type (WT), showing for the first time that ion channel activity of E protein is a determinant of CoV pathogenesis.\textsuperscript{59} The precise mechanism by which SARS-CoV E protein channel activity contributes to virulence is still not clear, but it may be related to inflammasome activation and elevation of interleukin-1\( \beta \).\textsuperscript{59}

Also, during virion egress, CoV infection drives a rearrangement of host cell membranes including the Golgi complex,\textsuperscript{60} and virions appear in large vacuoles derived from Golgi/ER-Golgi intermediate compartment membranes.\textsuperscript{51} When IBV E residue Thr16 was mutated to Ala, this resulted in decreased Golgi disruption.\textsuperscript{52} Thr16 is aligned with SARS-CoV E Asn15 (Figure 1) and therefore is expected to abolish channel activity. Thus, in addition to its possible contribution to virion assembly, channel activity of E proteins may contribute to CoV virulence by disrupting the balance of luminal environments and subsequently affecting the secretory pathway, leading to release of virions.\textsuperscript{63} However, this disruptive effect could not be restored by conservative polar substitutions with Ser, Asn, or Gln;\textsuperscript{32} therefore, other context-dependent changes may be necessary to recover channel activity.

**Human RSV SH protein**

The human respiratory syncytial virus (hRSV) is an enveloped pneumovirus in the paramyxoviridae family that causes lower respiratory tract disease in infants, elderly, and immunocompromised populations worldwide.\textsuperscript{64} Up to 64 million reported cases of hRSV infection and 160,000 deaths occur each year. Although the virus was identified almost half a century ago,\textsuperscript{65} there are still no vaccines or effective antiviral drugs available.

The SH protein is one of the three RSV membrane proteins, along with F and G, which are key factors during virus attachment, fusion, and entry into host cells.\textsuperscript{66,67} The SH protein is a type II integral membrane protein 64 (RSV subgroup A) or 65 (RSV subgroup B) amino acids long, with a single \( \alpha \)-helical TM domain\textsuperscript{68,69} (Figure 3A) which is highly conserved.\textsuperscript{70,71} The C- and N-terminal extramembrane domains are oriented...
lumenally/extracellularly and cytoplasmically, respectively, that is, opposite to SARS-CoV E. Most SH protein accumulates at the membranes of the Golgi complex in infected cells, but it has also been detected in the endoplasmic reticulum and plasma membranes. During infection, the full-length unmodified form is the major species.

RSV lacking the SH gene (RSVΔSH) is viable, causes formation of syncytia, and grows as well as the WT virus in cell culture. However, RSVΔSH virus is attenuated and replicates tenfold less efficiently than the WT in the upper respiratory tract. RSVASH virus is also attenuated in vivo by virus passage in mouse and chimpanzee models. Overall, these results indicate involvement of SH protein in the pathogenesis of RSV infection.

In common to SARS-CoV E protein, SH protein blocks or delays apoptosis in infected cells. This protective effect is also observed in homologs of RSV SH protein (Figure 3A), that is, parainfluenza virus 5, mumps virus, and

---

**Figure 3** Sequences of hRSV SH protein and homologs in paramyxoviruses.

Notes: (A) Alignment of sequences of hRSV SH protein and homologs, showing the TM domain (pink) and the prevalence of C-terminal Histidine residues, with a 3–4 residue periodicity; (B) pore profile through the pentameric bundle of RSV SH protein; (C) overlay of the monomeric structure obtained in DHPC/DLPC bicelles (blue) and DPC micelles (red); some side chains are indicated for comparison of the two structures; (D) model for the pentameric bundle of SH protein where each monomer is colored differently. (B) and (C) This research was originally published in The Journal of Biological Chemistry. Gan SW, Tan E, Lin X, et al. The small hydrophobic protein of the human respiratory syncytial virus forms pentameric ion channels. J Biol Chem. 2012;287(29):24671–24689. © the American Society for Biochemistry and Molecular Biology. (C) Copyright © American Society for Microbiology, Journal of Virology, 2014;88(20):11899–11914, 10.1128/JVI.00839-14.

Abbreviations: hRSV, human respiratory syncytial virus; SH, small hydrophobic; TM, transmembrane; RSv, respiratory syncytial virus; DPC, dodecylphosphocholine.
J paramyxovirus. Recent reports have also suggested that SH protein activates the NLRP3 inflammasome.

**Structure of RSV SH protein**

Earlier cross-linking studies showed that SH protein form different oligomers in sodium dodecyl sulphate, including dimers, trimers, tetramers, and pentamers. However, both synthetic TM domain (residues 18–43) and full-length SH protein have been shown to form homopentamers in a variety of detergents. These oligomers may have been responsible for early reports showing increased entry in bacteria of low molecular weight compounds after SH protein expression.

---

**Figure 4** Binding sites of inhibitors in SARS-CoV E and SH proteins. (A) Superposition of TROSY-HSQC spectra of uniformly 15N-labeled SARS-CoV E in the absence (red) and presence (blue) of 0.4 mM HMA (shown above image A). Peaks that undergo significant shifts upon complex formation are highlighted; (B) residues showing larger and smaller shifts are represented in red and yellow, respectively; (C) same as (A) for uniformly 15N-labeled SH protein in the absence (red) and presence (blue) of 4.8 mM pyronin B (shown above image C). (D) Residues showing larger and smaller shifts are represented in red and yellow, respectively.


**Abbreviations:** SARS-CoV, severe acute respiratory syndrome coronavirus; SH, small hydrophobic; HMA, hexamethylene amiloride; TROSY-HSQC, transverse relaxation-optimized spectroscopy-heteronuclear single quantum coherence.
The mutual orientation of monomers in the TM pentamer was determined using site-specific infrared dichroism of isotopically labeled peptides in lipid bilayers. hRSV SH protein has two Histidine (His) residues, His22 and His51 (Figure 3A). These structural studies showed His22 to be in a luminal, close to interhelical, orientation. This orientation was later confirmed by NMR studies that reconstructed the pentameric α-helical bundle of the full-length protein in DPC micelles. Like in SARS-CoV E, the pore lumen is more open toward the cytoplasmic side (Figure 3B). In this “micelle” model, a single α-helical TM domain was flanked N-terminally by an α-helix, and C-terminally by an extended β-hairpin. Later, the structure of these extramembrane domains has been confirmed using a DHPC/DLPC (1,2-Dihexanoyl-sn-Glycero-3-Phosphocholine/1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine) bicellar system. However, in that “bicelle” model, the TM α-helix was extended one more turn, until the other His residue, His51, whereas Pro-58 is located at the tip of a C-terminal loop (Figure 3C).

Using the mutual orientations between TM domains in lipidic membranes and DPC micelles, the pentameric model of the SH protein channel was reconstructed (Figure 3D). According to this model, His51 side chains are facing the lumen of the channel, whereas His22 is slightly skewed toward the other helix–helix interface.

**Channel activity of RSV SH protein**

The contribution to channel activity and selectivity of these two His residues was examined using BLMs and purified SH protein. Although the overall conductance was pH-dependent, consistent with previous patch clamp reports, SH protein showed lower conductance, not activation, at low pH. Indeed, conductance was consistent with the titration of His residues, with less conductance at low pH, where both His should be protonated. Also, His residues are not required for channel activity, as the channel was active even when both His residues were changed to Phe. These results are consistent with a dramatic reduction of channel activity at lower pH observed previously for synthetic SH-TM (residues 18–43) in planar lipid bilayers, where only His22 was present. Overall, BLM results showed only minor variations in conductance and selectivity with pH. Similarly, ion selectivity was low, and appeared modulated by His51, with His22 contributing a more structural role. This is consistent with the observed electrophoretic pattern of His mutants; mutant H22A showed aberrant mobility and/or aggregation, whereas H51A showed mobility similar to the WT form. Poor in vitro ion selectivity has also been observed in other viroporins, for example, SARS-CoV E protein, or HCV p7, and it may be a hallmark of incomplete specialization of these channels.

**Channel activity inhibitors of SARS-CoV E and RSV SH proteins**

Availability of channel inhibitors would help in understanding the role of membrane permeabilization to ions in the viral life cycle of these viruses, and would also have obvious therapeutic potential. SARS-CoV E channel activity is inhibited partially by HMA in the $K_d \sim 10 \mu M$ range. The interaction of this drug with the channel has been mapped to residues located at the N- and C-terminal ends of the TM region (Figure 4A and B). Affected residues at the N-terminal end include the conserved polar residue Asn-15, suggesting HMA binding to the N-terminal, luminal facing, end of the channel.

RSV SH protein is partially inhibited by pyronin B with a binding affinity ($K_d$) of $\sim 7 \mu M$. Although modest, this $K_d$ compares favorably with the 16 $\mu M$ for amantadine inhibition of influenza M2 protein, the $\sim 10 \mu M$ reported for HMA inhibition of SARS-CoV E, 50–100 $\mu M$ for rimantadine inhibition of HCV p7, or the $>100 \mu M$ of HMA for p7 inhibition. SH protein conductance changed from 0.3±0.07 nS in control conditions to 0.12±0.06 nS...
in the presence of pyronin B. As in the case of HMA for SARS-CoV E (Figure 4A and B), binding of pyronin B was mapped at both ends of the TM domain (Figure 4C and D). At the C-terminal end, the residue most affected was Ala39, and a group of nearby residues. Interestingly, most of these C-terminal juxtamembrane residues (residues 38–43) form a conserved motif in RSV SH protein “A_{39}LNKL_{45}”, suggesting that binding of the drug may not only alter channel activity. This binding site is located at the luminal-facing narrowest region of the channel lumen (Figure 3B). By comparison, residues affected at the N-terminal end are not conserved. Pyronin B was found to bind SH protein mostly at the lipid-facing side of the TM α-helices and not into the pore lumen. This mechanism of inhibition is thus probably allosteric, similar to that proposed for rimantadine binding to the TM domain of M2\(^{50}\) or rimantadine to HCV p7.\(^{50}\)

**Conclusion**

In summary, despite the sequences of SARS-CoV E and hRSV SH protein not being evolutionarily related, they share remarkable structural and functional features. Both viroporins have a single α-helical TM domain that forms pentameric oligomers. These oligomers are responsible for channel activity that is not selective. In both cases, the TM α-helix is connected to a short cytoplasmic membrane-bound α-helix by a flexible loop (Figure 5). The lumen of the pore has a funnel-shaped structure, more open toward the cytoplasmic side. The luminal side is narrower and contains conserved residues that may be important for channel activity, that is, Asn-15 in SARS-CoV E and His51 in RSV SH protein. This narrower luminal end is also where inhibitors were found to bind.\(^{48,84}\) This funnel-like architecture has also been observed in other viroporins, for example, influenza M2 protein\(^{4}\) and HCV p7.\(^{50}\)

Both viroporins delay apoptosis in infected cells, which may help evade host inflammatory responses and the premature death of the host cells. However, their presence increases pathogenicity. The relation of channel activity with virulence has been established at least for SARS-CoV E. Hence, viroporins constitute an as yet untapped potential source of pharmacological targets.

In addition to this channel activity, both E and SH proteins have been found to participate in a number of interactions with viral and host proteins that are not the subject of this article, for example, SARS-CoV E interacts with viral nonstructural protein 3,\(^{3,87}\) M protein,\(^{29,88–90}\) or host PALS1,\(^{88}\) whereas SH protein interacts with viral G protein.\(^{89,90}\) These interactions probably contribute to viral morphogenesis and viral particle formation and participating in the entry or release of viral particles into or from cells.

**Acknowledgments**

This work has been funded by the Singapore National Research Foundation under CRP Award No NRF-CRP4-2008-02 and Tier 1 grant RG 51/13.

**Author contributions**

All authors contributed to conception and design, acquisition of data, or analysis, and interpretation of data; drafting the article or revising it critically for intellectual content; and final approval of the version to be published.

**Disclosure**

The authors report no conflicts of interest in this work.

**References**


