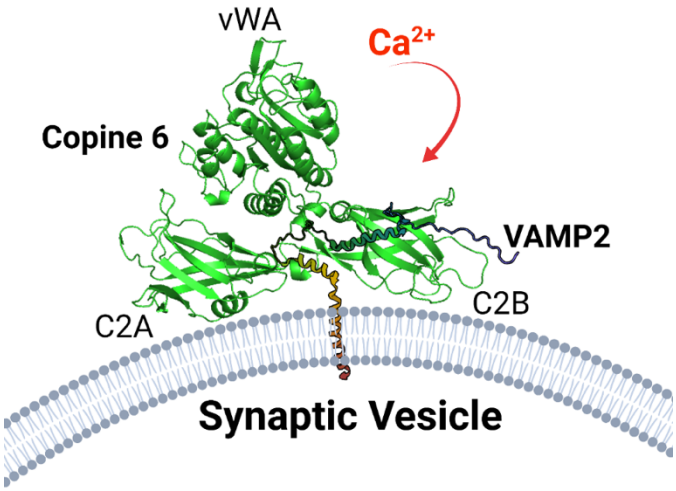
Converging mechanisms for calcium regulation of SNARE-dependent membrane fusion: EF-hand proteins and copines

Mehedi Hasan[1] and Mikhail Khvotchev[2,*]

[1] Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand; mehedihasan.saj@student.mahidol.edu
[2] Department of Biochemistry and Center for Neuroscience, Faculty of Science, Mahidol University, Bangkok, Thailand; mikhail.khv@mahidol.edu
* Correspondence: mikhail.khv@mahidol.edu

Abstract: Calcium-triggered membrane fusion is essential for nervous and endocrine system function and likely operates in many if not all nonneuronal cells. Similar to other types of membrane fusion, it critically depends on SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins, however SNARE protein function is not directly regulated by calcium. Instead, calcium activates specialized sensor proteins, which initiate or suppress membrane fusion by direct action on SNARE proteins. Broad range of calcium-membrane fusion coupling kinetics dictates the presence of multiple calcium sensors with distinct calcium-binding properties. Using a combination of in vitro biochemistry and bioinformatics we demonstrate that the juxtamembrane linker in the v-SNARE protein VAMP2 is highly conserved in evolution and is required for interaction with several calcium sensor proteins. We identify novel calcium-dependent VAMP2 linker-binding proteins that belong to EF-hand protein superfamily and build molecular models of VAMP2 complexes with C2 domain sensor protein copine 6 and EF-hand protein calmodulin. Thus, distinct calcium-binding proteins converge on the conserved juxtamembrane linker in VAMP2 and enable calcium regulation of VAMP2-dependent membrane fusion.

Graphical abstract:



Keywords: SNARE protein; calcium sensor; membrane fusion; synaptic transmission; molecular docking; EF-hand; C2 domain; copine

1. Introduction

Eukaryotic cells contain a complex network of internal membranes connected by tightly regulated membrane fusion events. Membrane fusion is orchestrated by multiple protein and lipid factors that govern donor and acceptor membrane proximity, lipid layer mixing, speed, precision, and specificity. SNARE proteins, conserved from yeast to humans, are thought to be universally required for all membrane fusion reactions in all cells [1]. SNARE proteins residing on opposing membranes, spontaneously assemble into tight four helical bundle complex and exert direct pulling force, thus executing membrane fusion [2]. Most membrane fusion reactions in cells are not tightly synchronized to a specific signal or stimulus and proceed constitutively. The notable exceptions are synaptic vesicle fusion at presynaptic terminals of neurons and regulated exocytosis of secretory vesicles found in endocrine and neuroendocrine cells where membrane fusion is initiated by and tightly coupled to a rapid rise in intracellular calcium concentration [3]. SNARE complex assembly is not sensitive to calcium, and requires calcium-binding proteins, or calcium sensors, that link SNARE proteins to calcium signal. Calcium sensors operate by activating membrane fusion machinery (e.g., destabilizing hemifused membrane intermediates) or by arresting membrane fusion followed by inhibition release by calcium. The most important and the best studied calcium sensor for synaptic vesicle fusion is synaptotagmin 1, a single pass membrane protein with tandem C2 domains that cooperatively bind multiple calcium ions [4]. The precise mechanism of synaptotagmin function is not yet resolved. The large set of structural and biochemical evidence suggests that synaptotagmin acts as a clamping sensor by direct or indirect interaction with SNARE complex [5]. In this model, calcium is not required for SNARE complex binding to synaptotagmin but disrupts this interaction by activating higher affinity binding of C2 domains to phospholipids in synaptic vesicle and plasma membranes. Next, the unclamped SNARE complex executes the final membrane fusion step. Synaptotagmin is a specialized calcium sensor that allows for tight spatiotemporal coupling of calcium influx and neurotransmitter release in synchronized synaptic transmission. Recent appreciation of synaptic output diversity and distinct forms of membrane fusion coupling with calcium signal in nonneuronal cells postulates additional calcium sensors that act at the earlier stages of SNARE complex assembly or on individual SNARE proteins. Previous studies identified calmodulin, as a calcium-dependent binding partner for v-SNARE protein VAMP2 [6]. In our recent work, we discovered that neuronal copine 6 interacts with VAMP2 in the presence of calcium and functions as a calcium-dependent regulator of spontaneous synaptic transmission [7]. Copine 6 belongs to the evolutionary conserved family of calcium-binding proteins comprised of tandem C2 domains and a vWA domain implicated in protein-protein interactions [8]. In the current study, we identified highly conserved linker region of VAMP2 that is essential for calcium sensor binding and performed an unbiased screen for calcium-dependent VAMP2 linker-binding proteins in the brain. Finally, we generated structural model for human neuronal copine 6 based on the recently elucidated 3D structure of plant copine protein BON1 and developed molecular docking models for VAMP2 linker complex with copine 6 and calmodulin.

2. Materials and Methods

Preparation of proteins and protein binding reactions. pGEX-KG plasmids encoding glutathione S-transferase (GST) fusion proteins with rat VAMP2 fragments were described previously or constructed using standard molecular biology procedures and verified by sequencing. Recombinant VAMP2 proteins were produced in BL21 E. coli strain and purified on glutathione–Sepharose beads (GE Healthcare). Stripped frozen rat brains (Pelfreeze) were homogenized in 150 mM NaCl, 25 mM HEPES, pH 7.4, 1 mM EDTA, protease inhibitor mixture Complete (Roche) and extracted by adding NP-40 to 2%. Brain extract was cleared by centrifugation. Recombinant bovine calmodulin was obtained from Merck Millipore. For pulldown reactions, GST beads were incubated with brain extract

(10 mg total protein per reaction) or recombinant calmodulin (5 mcg per reaction) for 3h at 4 ºC in the presence of calcium (2 mM $CaCl_2$) or in the absence of calcium (0.1 mM EGTA). Following extensive washing bound material was eluted from beads with 0.5M NaCl, 25 mM HEPES pH 7.4, 2 mM EGTA, 2mM EDTA and analyzed by SDS-PAGE. For protein identification, gels were silver stained, and individual bands were excised and analyzed by trypsin digestion/LC mass spectrometry (University of Texas Southwestern proteomics core facility). Bound calmodulin was visualized by Coomassie G250 gel staining.

Protein structure modeling and molecular docking. We obtained molecular model of human copine 6 using SWISS-MODEL resource (https://swissmodel.expasy.org) [9]. Human neuronal Copine-6 protein sequence (aa. 1-557) was retrieved from NCBI protein database (NP_001267487.1) and used as a query. 3D structure model of human copine 6 was generated for aa. 14-538 based on the experimentally determined plant copine BON1 structure, template: 6kxk.3.A, Q941L3 [10]. 3D model of copine 6 passed internal quality checks and was additionally verified for compliance with the Ramachandran plot by PROCHECK [11] (https://saves.mbi.ucla.edu). For molecular docking analyses, VAMP2 3D structure in lipid micelles was retrieved from RCSB PDB (2KOG), (https://www.rcsb.org/). The juxtamembrane region of VAMP2 (aa 80-94) as a ligand was docked with the newly generated copine 6 3D model and calcium-bound calmodulin 3D structure (PDB: 1OSA) using HADDOCK server [12] (https://bianca.science.uu.nl/haddock2.4). PDB structures of the ligand (VAMP2) and the receptor (copine 6 or CaM) were uploaded along with the active and passive residue information obtained from CPORT server [13] (http://alcazar.science.uu.nl/services/CPORT). Default docking parameters were used. Intermolecular complex clusters generated by HADDOCK were assessed using HADDOCK scores, cluster size, and several thermodynamic criteria. Model structures of each complex were analyzed for protein binding free energy by PRODIGY server [14] (https://wenmr.science.uu.nl/prodigy). Binding hot spots for both protein complexes were extracted by SPOTON server [15] (https://alcazar.science.uu.nl/cgi/services/SPOTON/spoton) and visualized by PyMoL.

3. Results

Conserved juxtamembrane linker in the v-SNARE protein VAMP2. VAMP2 protein contains 4 distinct regions: unstructured N-terminal proline-rich region of unknown function, SNARE motif that forms extended alpha-helix in SNARE complex, juxtamembrane linker and transmembrane domain (Figure 1). Mutations is the linker region have been shown previously to destabilize VAMP2 interaction with several calcium sensors [7, 16, 17] and with phospholipid membranes [18]. We analyzed the evolutionary conservation of VAMP2 linker in multiple species from human to round worms (Figure 1). In most warm-blooded vertebrate species, the linker region had no variation, except for several bird species that carry a single L93C substitution. Remarkably, evolutionary distant round worm *C. elegans* also carried a single L93I substitution. This position in VAMP2 varied the most across the species (L to C, M, A, I), importantly many observed substitutions were synonymous. The linker region contains a pair of vicinal tryptophan residues, that are conserved in all tested species.

VAMP2 linker is required for calcium-dependent interaction with multiple EF-hand proteins. To perform an unbiased search for calcium dependent VAMP2 interacting proteins we immobilized various fragments of human VAMP2 fused to GST on glutathione sepharose affinity sorbent. The longest fragment (2-94) contained the complete cytoplasmic sequence of VAMP2, and the shortest fragment (2-84) lacked the juxtamembrane linker. We applied total brain extract in the presence of calcium and eluted bound proteins by substituting calcium with EGTA followed by LC-MS identification of most prominent bands (Figure 2). We identified several EF-hand proteins including centrin 2, calmodulin and S100 proteins (S100A1 and S100B isoforms) that show robust binding to the full-length cytoplasmic fragment of VAMP2 (2-94) and reduced binding to a fragment truncated by 3 amino acid

residues (2-91). No biding to the EF-hand proteins was observed with shorter fragments of VAMP2. We did not detect any binding to VAMP2 fragments in calcium-free buffers (data not shown). To confirm that the VAMP2-EF hand protein interaction is direct, we performed the same experiment using recombinant calmodulin as a starting material. Similar to the previous experiment, strong binding was observed with 2-94 fragment, reduced binding with 2-91 fragment and no biding with the shorter fragments (Figure 2). Again, no binding to any fragment was observed in the absence of calcium (data not shown).

Modeling of VAMP2/Copine 6 and VAMP2/Ca^{2+}-calmodulin complexes. To gain insight into structural determinants of VAMP2 complexes we docked linker region of VAMP2 onto human copine 6 structural model and calcium-bound calmodulin. We extracted VAMP2 linker structural information from the previously determined NMR structure of VAMP2 in lipid micelles [19]. Currently, 3D structure for single copine protein from plants (BON1) has been determined [10]. BON1 share 50% identity with human copine 1 and 38% identity with human copine 6. We built human copine 6 structural model using Swiss-model resource (Figure 3a, b). The model shows triangular-shaped protein with C2A and vWA domains in close proximity and with C2B domain separated from vWA domain by a groove. VAMP2 linker region was docked into C2B-vWA groove with the N-terminal binding interface residues contacting vWA domain and C-terminal residues contacting C2B domain (Figure 3b). The docking model predicted several hydrophobic and basic amino acids in the linker region to form the binding surface (Table 1). Importantly, vicinal tryptophan residues are included in the binding interface. We also docked the VAMP2 linker region onto calcium bound calmodulin structure (Figure 3c, d). The predicted docking site includes the exposed surface of the N-terminal EF-hand domain and the extended alpha-helix that connect the N- and C-terminal domains. According to the docking model, C-terminal part of the VAMP2 linker region form contacts with the connecting alpha-helix and the N-terminal parts contacts with the N-terminal EF-domain of calmodulin (Figure 3d, Table 1).

3.2. Figures and tables

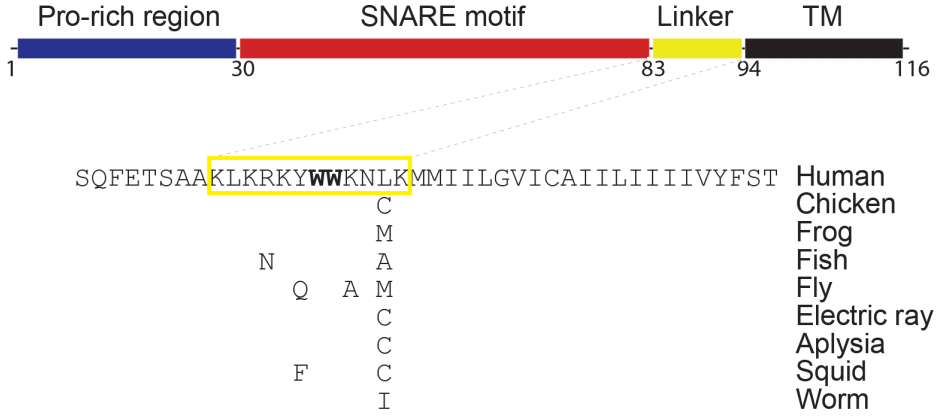


Figure 1. Structure of the v-SNARE protein VAMP2 (synaptobrevin 2) and the conserved juxtamembrane linker region. VAMP2 is divided into 4 regions: unstructured N-terminal proline-rich region, SNARE motif, juxtamembrane linker and transmembrane region. Amino acid numbering and sequence is indicated for human VAMP2. Linker region is boxed in yellow and vicinal tryptophan residues are shown in bold. For other species, only divergent amino acid residues within the linker region are shown.

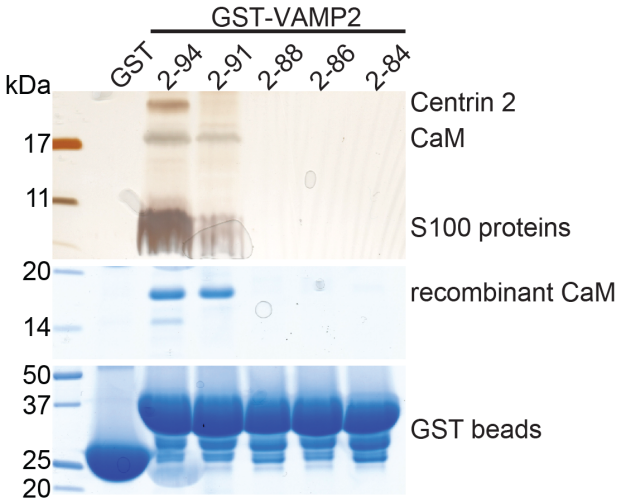


Figure 2. EF-hand proteins bind to the juxtamembrane region of v-SNARE protein VAMP2 in the presence of calcium. Upper panel shows pulldown experiment using total brain extract (silver stained SDS-PAGE gel), middle panel shows pull-down of recombinant calmodulin and lower panel shows proteins immobilized on glutathione Sepharose (both are Coomassie G250 stained). Experiments were performed in the presence of calcium. Immobilized proteins are noted on the top with GST alone serving as a negative control. Pulled down proteins are identified on the right and positions of protein molecular wight marker bands are shown on the left.

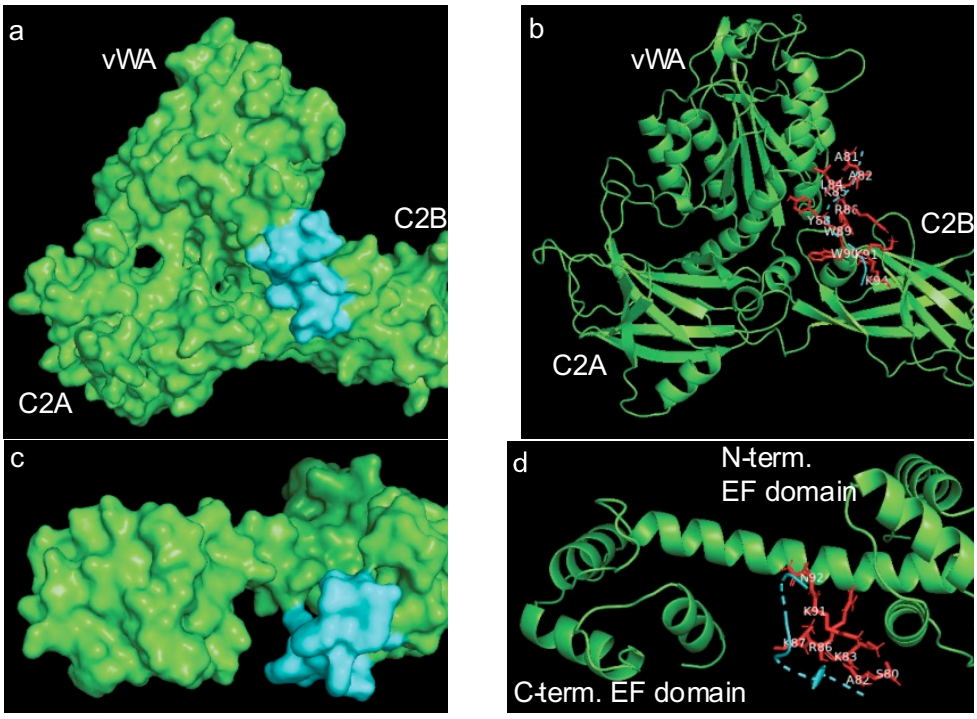


Figure 3. Structures of molecularly docked protein complexes formed by v-SNARE protein VAMP2 and the calcium-binding proteins copine 6 (a, b) and calmodulin (c, d). Space-fill models are shown in a and c, ribbon models in b and d. The receptor (copine 6 and calmodulin) is shown in green, the ligand (the juxtamembrane region of VAMP2) is in light blue. Critical amino acid residues in VAMP2 that form the binding interface are shown in red (summarized in the Table 1). The numbering corresponds to human VAMP2 protein. Individual domains of copine 6 (C2A, C2B and vWA) and calmodulin (the N- and the C-terminal EF hand domains) are indicated.

Table 1. VAMP2 binding interface in the molecularly docked protein complexes.

Protein complex	Binding free energy (Kcal/mol)	Critical amino acid residues in VAMP2 binding interface
Copine 6/VAMP2	-7.8	A81, A82, L84, K85, R86, Y88, W89, W90, K91, K94
Calmodulin/VAMP2	-4.1	S80, A82, R86, K87, K91, N92

4. Discussion

Calcium-triggered membrane fusion depends on multiple calcium sensors that allow for diverse kinetic coupling of the stimulus and the membrane fusion event [3]. Several calcium sensors, known to date, appear to associate with the fusion machinery via the juxtamembrane region of v-SNARE protein VAMP2. Mutations in this region are known to disrupt calcium sensor binding and function [7, 16, 20]. Mechanistically, it is not surprising that v-SNARE protein found on the donor membranes serves as a focal point for calcium sensor integration. In addition, localization of the binding interface to the juxtamembrane region of VAMP2 enables initial SNARE complex formation. To gain insight into VAMP2 linker role, we analyzed the evolutionary conservation of its sequence. Usually, linker sequences separating functionally defined regions in proteins are not highly conserved. Surprisingly, the juxtamembrane linker in VAMP2 is invariant in most analyzed vertebrate species and few, mostly synonymous substitutions are found in evolutionary distant organisms such as C. elegans (Figure 1). In addition, a pair of vicinal tryptophan residues are invariant in all known VAMP2 homologs. This observation suggests an important conserved function for the VAMP2 linker. The juxtamembrane linker structure is preserved in several v-SNARE proteins, from closely related VAMP1 and VAMP3 to more distant VAMP8 and VAMP4 (data not shown).

To identify novel calcium sensor candidates, we performed an unbiased screen of brain proteins using the soluble cytoplasmic fragment of VAMP2 as a bait. We searched for calcium sensors that fulfill two criteria: a) they bind to VAMP2 only in the presence of calcium and b) they require intact juxtamembrane linker for binding to VAMP2. Our screen is not exhaustive because these criteria apply only to a subset of functional calcium sensors. Complexin and synaptotagmin appear to interact with SNARE complex independent of calcium. Calcium acts at a later stage by initiating the phospholipid binding of C2 domains [5]. We identified several EF-hand calcium-binding proteins in our search (Figure 2). Calmodulin species have been shown to interact with VAMP2 [6]. In addition, we discovered novel EF-hand interacting proteins including centrin 2 and several S100 protein isoforms. Previously, 'calcium-independent' isoform S10010A was shown to interact with VAMP2 [21]. In our experiments, centrin 2 binding appeared to be the most sensitive to the presence of intact VAMP2 linker region. Removal of 3 amino acids from the C-terminus of VAMP2 (2-91 fragment) abolished the interaction with centrin 2 and significantly weakened the interaction with calmodulin and S100 proteins. Additional truncations in the linker sequence abolished the interaction with all EF-hand proteins. Notably, in these truncated fragments vicinal tryptophan residues were removed (WW89,90). WW mutations have been shown previously to abolish binding of complexin/synaptotagmin, calmodulin and copine 6 [7, 16, 17]. Finally, we confirmed that EF-hand proteins interact with VAMP2 directly using recombinant purified calmodulin in binding reactions with recombinant purified VAMP2 fragments. We did not test other EF-hand proteins; however, it appears likely they also bind VAMP2 directly.

To investigate molecular structure of the identified VAMP2 complexes, we took advantage of the structural information already available for VAMP2 and calcium-bound calmodulin. Monomeric VAMP2 is a relatively disorganized protein, therefore we employed the NMR 3D structure of VAMP2 in lipid micelles [19]. We extracted the structural information for the extended linker sequence, aa. 80-94, which includes alpha-helical and

disorganized elements. It seems likely that the critical binding interface should reside withing the linker sequence, however we cannot exclude the possibility of additional contact sites elsewhere in the structure of VAMP2. No 3D structural information is currently available for any animal copine. Therefore, we modeled 3D structure of human copine 6 using recently determined structure of BON1, plant copine protein [10]. Human copine 6 shares 38% sequence identity with plant BON1, however, bulk of the protein is comprised of 3 conserved domains, C2A, C2B and vWA, each of which is involved in calcium regulation. BON1 was crystallized without calcium or calcium chelators. In addition, protein displayed significant conformation flexibility in crystal units. Therefore, we hypothesized that the reported 3D structure of BON1 represents calcium-stabilized conformation of the protein. Generated molecular docking model predicts that VAMP2 linker binds in the groove separating vWA and C2B domains (Figure 3a, b). This is an agreement with our previous observations that VAMP2 binds full-length copine 6 but not C2AB or vWA domains separately [7]. VAMP2 binding interface includes the 2/3 of linker region and both vicinal tryptophan residues (Table 1). We docked VAMP2 linker region with the N-terminal EF-hand domain and extended alpha-helical connector of calcium-bound calmodulin (Figure 3c, d). This area is reported to form typical Ca^{2+}-calmodulin binding site for other proteins [22], however only the 1/3 of linker region and none of vicinal tryptophan residues are included in the binding interface in our model. Notably, S100 proteins, that we identified as binding partners for VAMP2, lack the C-terminal EF-hand domain.

Our study raises important questions about calcium sensor integration into SNARE-mediated membrane fusion. Do the novel VAMP2-binding proteins we describe here operate as in vivo calcium sensors and for what type of calcium-triggered membrane fusion reactions? Do they work by activating or inhibiting membrane fusion? Do other v-SNARE proteins that have similar juxtamembrane linker regions employ similar mechanisms of calcium regulation? We hope to address some of these unresolved questions in our future work.

5. Conclusions

The v-SNARE protein VAMP2 participates in several calcium-triggered membrane fusion reactions that enable synaptic transmission in neurons and hormone release in endocrine cells. Conserved juxtamembrane linker in VAMP2 serves as a converging focal point for multiple calcium sensors with divergent calcium-binding mechanisms and distinct calcium binding kinetics. Similar mechanism of calcium regulation may operate in other v-SNARE proteins that share basic architecture of the linker region.

Author Contributions: Conceptualization, MH and MK; methodology, MH and MK.; software, MH.; validation, MH and MK.; formal analysis, MH and MK.; investigation, MH and MK; resources, MK.; data curation, MH and MK.; writing—original draft preparation, MH and MK.; writing—review and editing, MK.; visualization, MH and MK.; supervision, MK.; project administration, MK. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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