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The role of TNFR2+ Tregs in COVID-19: An overview and a potential therapeutic strategy

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ABSTRACT

COVID-19 is a multi-faceted disease ranging from asymptomatic to severely ill condition that primarily affects the lungs and could advance to other organs as well. Its causing factor, SARS-CoV-2 is recognized to develop robust cell-mediated immunity that responsible to either control or exaggerate the infection. As an important cell subset that control immune responses and are significantly dysregulated in COVID-19, Tregs is proposed to be considered for COVID-19 management. Among its hallmark, TNFR2 is recently recognized to play important role in the function and survival of Tregs. This review gathers available TNFR2 agonists to directly target Tregs as a potential approach to overcome immune dysregulation that affect the severity in COVID-19. Furthermore, this review performs a rigid body docking of TNF-TNFR2 interaction and such interaction with TNFR2 agonist to predict the optimal targeting approach.

Invited review article

1. Introduction

Since declared a pandemic in January 2020, coronavirus disease 2019 (COVID-19) continued to cause thousands of new infection cases daily and more than two million deaths worldwide till March 2021 [1]. Its causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causes infection that produces a wide spectrum of manifestations, ranging from no symptoms to viral pneumonia leading to acute respiratory distress syndrome (ARDS) [2]. Hence, management of COVID-19 is based on symptoms experienced by patients, and not until recently, several vaccines have been approved to curb this pandemic [3].

SARS-CoV-2 enters host cells through binding of its spike (S) protein with host receptor angiotensin converting enzyme 2 (ACE2) which is abundant in lungs, and use human proteases such as transmembrane serine protease 2 (TMPRSS2) to activate the entry [4]. This feature is similar to entry of previous SARS-CoV that contribute to the rapid spread and wide range of symptoms and severity [5]. Upon entry and activation of SARS-CoV-2 infection, a cascade of immune responses involving both innate and adaptive immunity is induced, and the imbalanced of these responses dictate the extent of the COVID-19 development [6,7]. Available data demonstrated that infection of SARS-CoV-2 induces abnormally high activation of immune responses, which is non-effective in antiviral protection but instead lead to detrimental immunopathogenesis [8–11]. Thus, immune cells such as regulatory T cells (Tregs) that suppose to control these excessive immune responses should be highly considered in COVID-19 management.

This review proposes another appealing marker of Tregs, which is tumor necrosis factor receptor 2 (TNFR2), to be targeted in Tregs-based therapies for management of COVID-19. Furthermore, this review outlined the existing TNFR2 agonists as well as a potential approach to utilize them in overcoming cytokine storm, and thus reducing the severity of COVID-19. Furthermore, we performed rigid body docking of the interactions of TNFR2 agonists with TNFR2. A systematic search of a uniform sample of docked protein poses and uses an internal scoring algorithm to predict the optimal interactions. The following steps were performed: calculating docked protein poses, filtering docked protein poses for poses with specific residues at the binding interface, re-ranking docked protein poses with ZRank, as well as clustering docked protein poses and calculate density [12–14].
2. Regulation of Tregs in COVID-19

As a new disease, COVID-19 prompts progression into a severe state in a portion of patients continues to challenge its management and increase its morbidity and mortality. Later, it is recognized that the progression occurred in patients with existing immune dysregulation that developed into a condition known as ‘cytokine storm’ [15,16]. Cytokine storm involves a high accumulation of cytokines that mediate pro-inflammatory mechanism causing acute lung injury as well as multi-organ failure [17]. The major pro-inflammatory cytokines in this inflammatory reaction include interleukin-6 (IL-6), IL-1β, tumor necrosis factor (TNF-α) and interferon (IFN)γ [18]. In principle, our body defense towards uncontrolled inflammation is provided by a population of cells known as Tregs. Tregs function by suppressing these excessive effects of other cells in order to maintain immune homeostasis. Current shreds of evidence have demonstrated that Tregs is generally reduced in COVID-19 patients, particularly in severely ill patients [19–23]. Furthermore, dysregulation of Tregs displays a prominent identification that could serve as a prognostic marker in severely-ill patients [24].

However, there are several conflicting observations in the regulation of Tregs in COVID-19 (Table 1). Chen et al. [29] reports an increased in Tregs and one of its mediator, IL-10, in the peripheral blood of COVID-19 patients. Tregs is demonstrated to be amplified in both mild and severe cases, while IL-10 is elevated in severe cases only. This trend is similar to the immune profile in children with COVID-19, which was reported to show mild symptoms and had a better prognosis than adult counterpart [19]. Another study reported that Tregs is increased whereas Th1, Th2 and Th17 cells are decreased in patients with COVID-19 compared to healthy individuals [26]. A preprint also demonstrated an increase of Tregs number and its fork head box P3 (Foxp3) marker in COVID-19 patients, which directly correlates with poor patient outcomes [27]. Accordingly, these Tregs over-expressed both suppressive and pro-inflammatory effectors, suggesting to the faulty role of Tregs in the progression of COVID-19. It is noted that these increased of Tregs is accompanied by the decrease of T cells. Another study observed an increased pool of Tregs, associated with non-survivor severe cases is SARS-CoV-2-specific, which hindered the proliferation of effector T cells (Teffs) that contribute to recovery from COVID-19 [28]. This observation supports the idea of suppressive function of Tregs that inhibit the excessive effects of other cells in order to maintain immune homeostasis. Current shreds of evidence have demonstrated that Tregs is generally reduced in COVID-19 patients, particularly in severely ill patients [19–23]. Furthermore, dysregulation of Tregs displays a prominent identification that could serve as a prognostic marker in severely-ill patients [24].

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3. Role of TNFR2 on Tregs

Tumor necrosis factor (TNF) is considered an essential pleiotropic cytokine in orchestrating various immune reactions, development of diseases as well as cell survival and proliferation. TNF triggers a rapid and robust immune response in host defense against pathogens, while limiting the extension of the inflammatory response when the invasion is subsided or resolved [32]. It stimulates the production of pro-inflammatory cytokines such as IL-6, IL-8, chemokines and even itself [33]. TNF-α exerts its pro-inflammatory function by recruiting immune cells including lymphocytes, monocytes, and neutrophils to the sites of inflammation [34]. In addition to apoptosis, TNF can also induce necrotic cell death under specific conditions [35].

Macrophages and monocytes are major producer of TNF and intriguingly, these cells are highly responsive to this cytokine. TNF is produced at low level by other cells, including several subsets of T cells, natural killer cells, dendritic cells, B cells, and astrocyte [36]. TNF is a type II transmembrane protein, a stable form of 233 amino acid homotrimers [37]. This cytokine primarily generated in a form of membrane-integrated protein (mTNF) with relative molecular weight at 26 kDa. From this bounded protein, the homotrimeric TNF is processed and released in a 17 kDa trimeric soluble form (sTNF) via the proteolytic activity of metalloprotease TNF alpha converting enzyme (TACE) [38]. Both forms of TNF are biologically active non-covalent bonded homotrimers. Despite of that, the nature of specific functions of mTNF and sTNF remains unclear.

TNF execute its functions through interaction with two distinct transmembrane receptors; TNFR1 (CD120a; p55/60, 55 kDa) and TNFR2 (CD120b; p75/80, 75 kDa) [39]. Each receptor has an extracellular binding region, a transmembrane segment, and intracellular transmembrane receptors; TNFR1 (CD120a; p55/60, 55 kDa) and TNFR2 (CD120b; p75/80, 75 kDa) [39]. Each receptor has an extracellular binding region, a transmembrane segment, and intracellular region. Both TNFRs have similar multiple cysteine-rich motif in the extracellular region and are active in form of homodimers [40]. As the main receptor to the mTNF, TNFR2 forms a tight trimer and binds stronger than to sTNF [40]. The specific binding pattern between TNF and TNFR2 has been only revealed recently by Mukai’s group [41]. The interaction between TNF and TNFR2 has been identified to potentially regulate the activity of Tregs in COVID-19 patients with severe COVID-19 infection (Fig. 1).

Previously, Stephen-Victor et al. [31] had reviewed the potential of Tregs-based therapies in the management of COVID-19. Besides adoptive transfer of ex vivo Tregs, they outline several other strategies to enhance Tregs through its stimulatory markers including CD25 and cytotoxic T-lymphocyte antigen-4 (CTLA-4). Based on the clinical benefits of these strategies in autoimmune and inflammatory diseases, they appear to be attractive to overcome hyperinflammation in severe cases of COVID-19. However, the disparities of Tregs among patients with COVID-19 demand for careful evaluation of Tregs phenotype, thus exact targeting of this cell subset could be applied.

Table 1: Regulation of Tregs in patients with COVID-19.

| Regulation of Tregs | Sample (n) | Stage | Definition of Tregs |
|---------------------|-----------|-------|---------------------|
| Downregulated       |           |       |                     |
| [25]                | PB (30)   | NR    | CD4+CD25+Foxp3+     |
| [24]                | PB (19)   | Severe| CD3+CD25+CD127+     |
| [20]                | PB (109)  | Mild  | CD25+Foxp3+         |
| [23]                | PBMC (40) | Severe| CD4+CD25+CD127-     |
| [19]                | PB (15)   |       | Gene expression of Foxp3, IL-10, TGF-β | |
| [26]                | PB (57)   | Recovery| CD127+Foxp3+       |
| [27]                | PB (57)   | Severe | CD25+Foxp3+         |
| [11]                | PB (6)    |       | CD45RO+CD45RA+CD25+CD125low |
| [28]                | PBMC (33) |       |                     |
| [29]                | PB (80)   |       |                     |
| [22]                | PB (19)   |       |                     |

NR, not reported; PB, peripheral blood; PBMC, peripheral blood mononuclear cells.
occur in two TNF-binding regions of TNFR2 monomer, namely, region 3 and 4 (Fig. 2). To better understand the respective view of the two binding regions to the overall binding, we performed a molecular dynamic simulation of the TNF–TNFR2 interaction using Discovery Studio (PDB ID 3alq). The molecular surface of region 3 of TNFR2 is a highly negatively charged, made up by three acidic residues such as Asp54, Glu57, and Glu70. Meanwhile, region 4, conversely, contains three basic residues, such as Arg77, Lys108, and Arg113, which together create highly positively charged molecular surface [42]. The binding strength of TNF in region 3 is relatively weaker than that of in region 4. This could lead to more competitive ligand binds against TNF on TNFR2, hence it may be more viable for small molecule inhibitor targeting region 3, although the inhibitory effect could be negligible. Targeting region 4 with small molecules inhibitor should potentially have higher inhibitory effect, however, since TNF binding to this region is stronger, the challenge in finding suitable molecules could also be greater [43].

### 3.1. TNF-TNFR2 interaction and its signaling pathways

Interaction of TNF with TNFR2 is illustrated in Fig. 2. Initially, TNFR2 was suggested to exist as the supporter to enhance TNFR1-induced cell death by a process called “ligand passing” [44]. However, recent investigations by engaging mutant forms of TNF or agonistic antibodies exclusively to TNFR1 or TNFR2 have further characterized that these receptors are receptor-specific ligands, suggesting the two TNFRs primarily activated via distinct signaling pathways. The binding of TNF to TNFR2 is extremely important for cell survival and proliferation [45]. Through binding to TNFR2, TNF triggers the intracellular domains of the receptor to recruit existing cytoplasmic TNF receptor-associated factor-2 (TRAF-2)-complex inhibitors of apoptosis proteins 1 (cIAP-1)-cIAP-2 complexes [46]. The ubiquitin-ligase activity of cIAP can suppress the pro-apoptotic activity by binding to the caspases and other apoptosis-inducing factors [36]. This leads to the activation of nuclear factor kappa B (NF-kB) which regulate the transcriptions of genes including those responsible for cell survival and anti-apoptotic gene pathways [46]. Other than NF-kB, in the same context, TNF-TNFR2 interaction stimulates a reciprocal PI3K/Akt pathway [47]. Moreover, the interaction recruits Etk, an endothelial/epithelial tyrosine kinase, to form the TNFR2-Etk–vascular endothelial growth factor receptor 2 (VEGFR2) complex that has been implicated in cell adhesion, migration and proliferation [48,49]. Of note, TNFR2 lacks a death domain. Unlike TNFR1 which mediates death signaling, TNFR2 interacts directly via its intracellular region with the adaptor protein TRAF-2 under certain conditions like stress or when the exhaustion of the cIAP pool, as such initiates the apoptosis pathway [50].

Previously, TNF is shown to be upregulated along with other pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ) in patients with COVID-19. Hence, the potential of anti-TNF therapies ought to be considered, and yet there are concerns regarding this approach, particularly its safety [51]. Anti-TNF is usually indicated in autoimmune diseases and effectively resolves the symptoms but there are several adverse effects such as severe infections are reported [52]. Thus, the use of this therapy needs to be approached with caution because it may increase viral replication or bacterial infection. Unlike its established pro-inflammatory activity in disease, TNF is also shown to modulate anti-inflammatory responses to viral infections and other infectious agents, through its interaction with Tregs [53,54]. These studies have sought to define the prominent role of TNFR2 in the potent immunosuppressive effects of Tregs. TNFR2 can only be efficiently activated by binding to mTNF [55]. Because of the higher affinity in binding, the bond is too stable to dissociate than sTNF [56]. While TNFR1 is predominantly expressed in nearly all cell types,
TNFR2 is strictly expressed on myeloid and lymphoid cell lineages, particularly on Tregs, and other non-immune cells (e.g. endothelial cells and neural cells) [50,57,58]. Due to these exclusive expressions, TNFR2 is responsible for mediating proliferation and maintenance of function in Tregs as well as Teffs [59,60]. Interestingly, preferential expression of TNFR2 on Tregs and its interaction with TNF directly dampen the inflammatory responses, as such, leads to high immunosuppressive efficiency and cell survival [38].

In detail, the interaction of TNF with TNFR2 generates costimulatory signals that able to enhance cell proliferation and survival in T cells [61]. Meanwhile, in the subpopulation of T cells, it has been established that TNFR2 signaling is directly associated with the phenotype and functional properties of Tregs, which predominantly expressed both in mice [62] and humans Tregs [63]. In TNFR2 deficient mice, thymic and peripheral Tregs populations were reduced [64] and inflammatory responses in vivo were dysregulated [62]. In human, thymic Tregs expressed a markedly higher level of TNFR2 compared to thymic Teffs [63]. Interestingly, TNF-TNFR2 ligation on Tregs exhibited the most potent suppressive capacity [64,65]. Furthermore, under pathological condition, TNF-TNFR2 interaction was found to down-regulate FoxP3, which is later blocked and restored by TNF antagonist [66,67]. Foxp3 is a transcription marker specifically required for regulate FoxP3, which is later blocked and restored by TNF antagonist [64,65]. Furthermore, under pathological condition, TNF-TNFR2 interaction was found to down-regulate FoxP3, which is later blocked and restored by TNF antagonist [66,67]. Foxp3 is a transcription marker specifically required for

activation of Tregs [68]. Both murine TNFR2+ and TNFR2− CD4+CD25+ T cells expressed a comparable high abundance of FoxP3 [62]. However, FoxP3 alone is intrinsically weak to activate the suppressive function in TNFR2− CD4+CD25+ T cells [62]. Unlike FoxP3, TNFR2 does not stimulate CD4+CD25+Foxp3+ T cells to become suppressive [68]. In contrast, TNFR2 in CD4+Foxp3− T cells exhibit higher resistance to suppression by CD4+CD25+ Tregs. Previously, a subpopulation of CD4+ T cells expressing CD25, the IL-2R alpha subunit, was suggested as an essential for functional programming of Tregs [69]. However, CD4+CD25+ TNFR2+ cells were shown to have greater suppressive capacity compared to CD4+CD25− TNFR2− cells, despite the cells expressed lesser FoxP3+ population than the latter, suggesting that expression of TNFR2 is considered to be more significant to suppressive phenotype in Tregs than CD25 [70]. Additionally, more functional suppressive Foxp3+ Tregs in human can be identified in co-expression of TNFR2 with CD25 [70]. It has also been reported that TNFR2+ Tregs preferentially accumulate in tumor more than in the periphery and this subset has shown to have more potent suppressive capacity [62]. The interaction of TNF-TNFR2 promotes both Tregs and Teffs proliferation; however, TNFR2 expressed on Tregs has shown to resist the inhibition of suppression of TNFR2+ Teffs [71]. Fig. 3 summarized signaling cascades upon interaction of TNF with TNFR2 and their preferential expression on Tregs that lead to its crucial role on this cell subset.

Other than CD4+ Tregs, CD8+ Tregs expansion is induced specifically via TNFR2 signaling by interaction with mTNF expressed by activated CD8+ T cells [72]. While CD4+ Tregs co-expression of CD25 and TNFR2 are notably recognized as a potent subpopulation of Tregs, CD8+ Tregs exhibits TNFR2 as the more important marker than CD25 [60,73]. From the latter, it seems that TNFR2 expression is the prominent checkmark of potent subpopulation of Tregs. Despite of that, TNF-TNFR2 axis has been demonstrated to diminish the functional suppressor activity of Tregs, one of them is through the activation of NF-κB signaling pathway, preferentially activated by pro-inflammatory TNFR1 [74]. This raised question of TNF-TNFR2 axis on the function of Tregs could be associated with the crosstalk of TNFR2 with TNFR1 [75]. Under certain conditions including prolonged cell stress in disease condition, TNFR2 can be shifted to TNFR1 apoptotic signaling, lead to the opposite of Tregs function [75]. Even though the crosstalk relationship between TNFR1 and TNFR2 has attracted conflicting findings and interpretations,

![Fig. 3. Signaling cascade of TNFR2. Activation of mTNF and TNFR2 triggers TRAF-clAP complexes that initiate both NF-κB and MAPK signaling pathways. NF-κB pathway activates IL-2 proliferation and reciprocal PI3K/Akt that inhibit Th17 differentiation and increased the phosphorylation of STAT5. The release of IL-10 and TGF-β along with IL-2 are associated with cell survival and proliferation as well as its function. Since TNFR2 is preferentially expressed on Tregs compared to other cells, activities from this signaling are prominently exhibit on this cell. Created with BioRender.com.](image-url)
TNFR2 remains a part of the anti-inflammatory feedback mechanism that promotes Tregs activity. This provides us a novel approach to regulate immune tolerance, thus prevent aberrant or excessive immune responses within the inflammatory environment. Likewise, it could be highly beneficial to use TNFR2 expressing Tregs as a therapeutic target in the management of COVID-19 patients.

4. Potential of targeting TNFR2 in COVID-19

Modulation of the TNF-TNFR2 axis emerged as a promising immunotherapeutic approach in several diseases or conditions that involve inflammatory reactions, including cancer, allergy, and microbial infections [76-81]. Previously, it is demonstrated that soluble TNFR2, along with TNFR1, is increased in COVID-19 patients and directly correlates with mortality for COVID-19 [82]. It is also observed that patients who recovered had a lower level of TNFR2 at hospital admission compared to those who ultimately died. Under inflammatory condition such as mycobacteria infection, shedding of soluble TNFR2 would occurred and neutralize TNF, in which would inhibit IL-6 production [83]. In a clinical trial of umbilical cord mesenchymal stem cell (UC-MSC) treatment in COVID-19 patients with ARDS, increased soluble TNFR2 is associated as the mechanistic role of reduced TNF and improved clinical outcomes of these patients [84]. Interestingly, this shedding into soluble TNFR2 is IL-10-dependent and in COVID-19, both IL-10 and IL-6 are significantly high [85]. In experimental coronavirus retinopathy, the same trend is observed when serum TNF and both of its receptors are increased. Furthermore, the upregulation of TNFR2 is accompanied by the decrease of nitric oxide [86]. In the same study, it is recognized that TNFR2 release from monocyte downregulates the nitric oxide. Nitric oxide is established as a mediator in anti-microbial and anti-inflammatory activities and its potential in COVID-19 is currently under clinical evaluations [87]. Furthermore, an in silico study has demonstrated that polymorphisms of TNFR2 gene in COVID-19 were determined to affect several miRNAs binding sites that play important role in immune regulation and lung damage repair [88]. Thus, it is assured enough to propose that the TNFR2 in COVID-19 is dysregulated and induces a cascade of devastating effects including increase of monocytes which are also increased in COVID-19, and downregulation of nitric oxide, further worsen the severity of patients in COVID-19. Wang et al. [89] and Zhou et al. [90] had thoroughly compiled evidences that demonstrated the effectiveness of targeting Tregs and TNFR2, respectively, using pharmacological agents such as dexamethasone, hydroxychloroquine, vitamin D3, adalimumab, thalidomide and cyclophosphamide to modulate the number and function of Tregs in the treatment of major diseases. Therefore, TNFR2 agonist serves as the answer to directly control Tregs that play an important role in the pathogenesis of COVID-19 (Fig. 4).

4.1. TNFR2 agonists

TNFR2 agonists are used to activate the TNF-TNFR2 signaling pathways by mimicking the activity of mTNF, and thus promoting cellular cascades that involve in controlling cell death and anti-inflammatory responses [91,92]. The first fully human TNFR2 (hTNFR2) agonist was developed by Fischer et al. [93] to ameliorate neurodegenerative processes in vitro. This genetically engineered agonist was developed based on a TNFR2-selective mutein in the single-chain TNFR2 (scTNFR2), fused to the trimerization domain tenascin C (TNC), and thus it is known as TNC-scTNFR2 [93]. The potential therapeutic effect of TNC-scTNFR2 was also studied in autoimmune and demyelinating diseases using human peripheral blood mononuclear cells (PBMCs) primary astrocytes [94]. It is reported that TNC-scTNFR2 induced T cell activation through the increased IL-2-dependent IFN-γ production, while Tregs count was increased in PBMCs culture, thus suggested a potential role in the downregulation of T cell immune responses [94]. The ciliary neurotrophic factor, which enhances the formation of myelin, was also induced, rescuing differentiated neuron cells from cell death induced by hydrogen peroxide [94]. Using a hTNFR2-transgenic mouse model, pharmacokinetic behavior and potential systemic responses of TNC-scTNFR2 were studied in vivo. TNC-scTNFR2 showed a greatly extended plasma half-life with no signs of systemic toxicity, indicating that it is well tolerated even at higher doses above the maximally tolerated dose in wild-type [94]. Later, Dong et al. [91] have developed a similar hTNFR2 agonist based on the Eps15-homology domain-containing protein 2 (known as EHD2-scTNFR2) to study the protective role of TNFR2 in neurodegeneration. EHD2-scTNFR2 was utilized to discover the involvement of TNFR2 signaling in controlling autoimmune and inflammatory diseases in vivo and in vitro [92]. Administration of EHD2-scTNFR2 to mice with collagen-induced arthritis resulted in an increased expansion of their Tregs, inducing anti-inflammatory responses that relieve the arthritis [92]. Furthermore, an in vitro study on mouse T cells with EHD2-scTNFR2 and/or IL-2 showed that Tregs were most efficiently expanded in the presence of EHD2-scTNFR2 [92]. In another study, treatment of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), with EHD2-scTNFR2 was demonstrated to alleviate the sensory and motor deficits [95]. This indicates that EHD2-scTNFR2 primarily promotes suppression of both demyelination and autoimmune reactions as TNFR2-dependent responses [95]. Fig. 5 illustrates the structural interaction of both EHD2-scTNFR2 and TNC-scTNFR2 to TNFR2.

Although TNFR2 agonists emerged as promising therapeutics to treat a variety of diseases, limited studies have used them so far. After more than 7 years of experiments, the production of TNFR2 agonistic antibody was successfully achieved by Faustman’s group, using BALB/c mice immunized with fragments of hTNFR2 protein [96]. Their in vitro studies showed that this type of antibody has the potential to increase the expression of TNFR2, thereby expanding Tregs and suppressing Teffs [96,97]. Thus, it could be beneficial to target this axis in order to modulate various inflammatory diseases. However, several challenges in the development process arise in such immune agonist therapies [98]. There are no approved agonistic antibodies as yet for clinical use, or even have entered phase III trials. This could be due to the absence of specific biophysical characters that can reproducibly predict that an antibody has agonist properties. While the full functional characterization is the only confident process to confirm that, this means a longer time of investigation is needed.
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CRediT authorship contribution statement

Conceptualization – SA, RM; Data curation – SA, LL, MAIA, MMH, WA; Roles/Writing – original draft – SA, LL, MAIA; Writing – review & editing- MMH, WA, RM; Software – MAIA, WA; Supervision & Funding acquisition – RM. All authors contributed to the paper and approved the submitted version.

Declaration of competing interest

The authors report no conflict of interest.

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