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Review

Neutrophil-endothelial interactions in respiratory syncytial virus bronchiolitis: An understudied aspect with a potential for prediction of severity of disease

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ABSTRACT

Respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) causes significant morbidity and mortality among young infants worldwide. It is currently widely accepted that neutrophil influx into the airways is a hallmark of the pathophysiology. However, the exact mechanism of neutrophil migration from the vasculature into the alveolar space in RSV LRTI has received little attention. Data shows that endothelial cells become activated upon RSV infection, driving a 'pro-adhesive state' for circulating neutrophils with upregulation of endothelial intercellular adhesion molecule-1 (ICAM-1). During RSV LRTI different subsets of immature and mature neutrophils are present in the bloodstream, that upregulate integrins lymphocyte-function associated antigen (LFA)-1 and macrophage (Mac)-1, serving as ICAM-1 ligands. An alveolar gradient of interleukin-8 may serve as a potent chemoattractant for circulating neutrophils. Neutrophils from lung aspirates of RSV-infected infants show further signs of inflammatory and migratory activation, while soluble endothelial cell adhesion molecules (sCAMs), such as sICAM-1, have become measurable in the systemic circulation. Whether these mechanisms are solely responsible for neutrophil migration into the alveolar space remains under debate. However, data indicate that the currently postulated neutrophil influx into the lungs should rather be regarded as a neutrophil efflux from the vasculature, involving substantial neutrophil-endothelial interactions. Molecular patterns of these interactions may be clinically useful to predict outcomes of RSV LRTI and deserve further study.

1. Introduction

Respiratory Syncytial Virus (RSV) also known as human orthopneumovirus in the family Pneumoviridae, is the leading cause of acute lower respiratory infections (LRTI), such as bronchiolitis and pneumonia, among infants [1]. Worldwide at least 3.4 million RSV LRTI episodes necessitate hospital admission of infants below 1 year of age with estimated fatality rates of 0.7 % in Western countries and 2.1 % in developing countries [1]. The course of RSV LRTI is clinically variable, and it remains a huge challenge to discern whether a patient has a high probability to develop a severe course of disease [2,3].

The initial step in the pathophysiology of RSV LRTI is the activation of airway epithelial cells (AECs), the primary targets of RSV [4,5]. AECs are activated through pattern-recognition receptors (PRRs), and produce chemoattractants and cytokines including interleukin (IL)-1, IL-8, tumor necrosis factor alpha (TNFα), and complement C5a, initiating the

Abbreviations and acronyms: AEC, airway epithelial cell; ALI, acute lung injury; Ang-1, Angiopoetin-1; Ang-2, Angiopoietin-2; BAL, broncho-alveolar lavage; CAM, cell adhesion molecule; CX3CR1, CX3 chemokine receptor-1; ELISA, enzyme-linked immuno sorbent assay; F-protein, Fusion protein; G-CSF, granulocyte colony stimulating factor; G-protein, binding glycoprotein; ICAM-1, intercellular adhesion molecule-1; HMVEC, human microvascular endothelial cell; HPAEC, human pulmonary artery endothelial cell; HSPG, heparansulfate proteoglycan; HUVEC, human umbilical vein endothelial cell; LFA-1, lymphocyte function associated antigen-1; Mac-1, Macrophage-1; mRNA, messenger ribonucleic acid; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; NPA, nasopharyngeal aspirate; ROS, reactive oxygen species; RSV, respiratory syncytial virus; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cellular adhesion molecule; VLA-4, very late antigen-4

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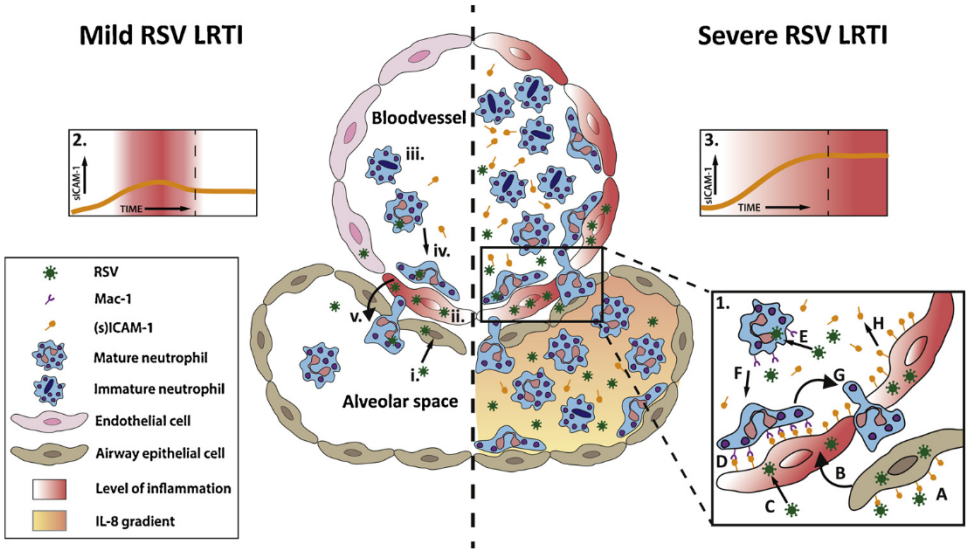

Fig. 1. Schematic representation of neutrophil-endothelial interactions during respiratory syncytial virus lower respiratory tract infection (RSV LRTI).
With this review, we have shown that during RSV LRTI airway epithelial cells (AECs) are infected with RSV (**i**), leading to activation of pulmonary vascular endothelial cell activation (**ii**), appearance of immature and progenitor neutrophils in the blood (**iii**), increased neutrophil-endothelial interactions (**iv**), and migration of neutrophils across the endothelium into the alveolar space (**v**) (**left panel**). Based on the available literature, we hypothesise that these features may occur to a lesser extent in mild RSV LRTI (**left panel**) *versus* severe RSV LRTI (**right panel**). A detailed description of the underlying mechanisms is given for severe RSV LRTI in **insert 1**. RSV directly infects airway epithelial cells (AECs) after binding of its Fusion protein with intercellular adhesion molecule-1 (ICAM-1) on AECs (**A**), followed by interleukin (IL)-1α signalling resulting in indirect activation of underlying endothelial cells (**B**). Endothelial cells are also directly infected with RSV (**C**). Endothelial cells upregulate ICAM-1 on their luminal cell surface (yellow), preparing a 'pro-adhesive' state for circulating neutrophils (**D**). Newly released immature and progenitor neutrophils and their mature counterparts can also become infected with RSV (E). An alveolar IL-8 gradient serves as a potent chemoattractant for circulating neutrophils (**right panel**). These neutrophils show increased cell surface expression of integrin Mac-1 (purple), the carbohydrate ligand of endothelial ICAM-1, which results in interactions with, and firm adhesion to, the endothelium (**F**) before migrating across the endothelium into the alveolar space (**G**). During these neutrophil-endothelial interactions, ICAM-1 is shed into the vasculature leading to increased levels of its soluble isoform sICAM-1 (**H**). Levels of sICAM-1 are low and resolving in mild RSV LRTI (**insert 2**), yet high and sustained in severe RSV LRTI (**insert 3**). ICAM-1 levels may be associated with more clinical symptoms in infected infants and deserve further study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

innate immune response [6]. During RSV LRTI, an influx of neutrophils into the airways occurs as shown in studies on post-mortem lung tissue or broncho-alveolar lavages (BAL) [7–10]. While this influx is often described as an innate immune response to infection, the exact molecular mechanisms involved in the migration of these neutrophils during RSV LRTI have received little attention. It has been well established in bacterial infection and acute respiratory distress syndrome (ARDS) with similar pulmonary neutrophil influx, that before extravasation and entry into the airways, neutrophils must undergo interactions with the pulmonary vascular endothelium. These interactions are orchestrated by cell adhesion molecules (CAMs) expressed on both cell types [11,12]. Soluble isoforms of these CAMs appear in the peripheral blood during RSV LRTI, implying a role for neutrophil-endothelial interactions in its pathophysiology [13–15] (Fig. 1).

In this narrative review, we summarize evidence of activation of endothelial cells and neutrophils, and of interaction of neutrophils with and migration across endothelial cells, occurring during RSV LRTI. Soluble CAMs may be useful to predict severity of RSV LRTI.

2. Review

2.1. Evidence of endothelial cell activation in RSV infection

2.1.1. Endothelial cell activation during RSV infection: towards a pro-adhesive state

During RSV bronchiolitis, AECs are the main initial targets of the virus, and are activated through recognition of RSV messenger ribonucleic acid (mRNA) and viral proteins by PRRs on their surface [4,5]. RSV glycoprotein (G-protein) and fusion glycoprotein (F-protein) play a major role in the initial phases of infection [16,17]. G-protein binds to the CX3C chemokine receptor 1 (CX3CR1), and F-protein binds to ICAM-1, both expressed on the surface of AECs. In a study by Behera et al., using different airway epithelial cell lines, it was shown that *in vitro* RSV infection of AECs is dependent on intracellular adhesion molecule-1 (ICAM-1), since blocking of ICAM-1 on the epithelial cell surface with neutralizing antibodies inhibited AEC infection with RSV [18]. In the same study, it was shown that blocking of the RSV fusion-protein (F-protein) suppressed infection of AECs, suggesting an important role for RSV F-protein ICAM-1 interactions in RSV infection of AEC's [18].

Chang et al. further investigated the role of ICAM-1 in RSV bronchiolitis [19]. In their study, it was hypothesized that, after the first encounter of RSV with AECs, RSV indirectly activates underlying vascular endothelial cells through signalling from AECs. *In vitro* cultured human umbilical vein endothelial cells (HUVECs) were exposed to either supernatants of human AECs infected with RSV (in which RSV viability was destroyed by UV radiation), or medium containing TNF-α as a positive control, and incubated for a total duration of 48 h. ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin were then measured in the supernatant of the HUVECs using enzyme-linked immunosorbent assay (ELISA). Endothelial cells that were exposed to supernatants of RSV-infected AEC showed levels of ICAM-1 that increased over time and, to a lesser extent, increased levels of E-selectin and VCAM-1, when compared to negative controls. Furthermore, selective blockage experiments showed that pre-treatment of RSV-infected AECs with an anti-IL-1α antibody significantly reduced ICAM-1 levels in endothelial cell supernatants. Antibodies against IL-1β and TNF-α did not induce this effect, which suggests that IL-1α is the predominant factor produced by infected AECs that activates endothelial cells. The temporal increase of ICAM-1 levels in the endothelial cell supernatant confirms that the ICAM-1 originates from shedding by endothelial cells, and not from accidental transfer by RSV-infected AEC's [19].

The levels of soluble CAMs, such as ICAM-1, are the result of cellular surface expression and subsequent cleavage by shedding enzymes, which are membrane-bound enzymes (sheddases) that cleave extracellular portions of transmembrane proteins [11]. The extent of ICAM-1 expression on endothelial cells during RSV-infection was assessed in a study by Arnold et al., in which the authors show that RSV can infect and directly activate endothelial cells [20]. The authors then further hypothesized that RSV infection favours an adhesive phenotype of *in vitro* cultured endothelial cells. HUVECs, human microvascular endothelial cells (HMVECs), and human pulmonary artery endothelial cells (HPAECs) were incubated with RSV at different concentrations. Endothelial cells were then fixed and stained for immunofluorescent

imaging and harvested for determination of cellular ICAM-1 mRNA with quantitative PCR. In both assays, an increased ICAM-1 expression was observed as early as 24 h post infection, still steadily increasing after 72 h. ICAM-1 expression on all endothelial cell lines was higher after incubation with higher concentrations of RSV virus. Interestingly, similar observations were done in HMVECs infected with influenza A H1N1 virus infection [21].

In summary, both indirect activation through AEC cell signalling and direct RSV infection of endothelial cells drives upregulation and release of CAMs, particularly ICAM-1. These molecular changes indicate a 'pro-adhesive state' of the vascular endothelium during RSV infection, probably as a prerequisite for leukocyte adhesion. These soluble endothelial adhesion molecules can be measured systemically.

2.1.2. Systemic levels of endothelial soluble adhesion molecules during RSV infection

During inflammation and infection, soluble isoforms of CAMs are released into the systemic bloodstream by enzymes called sheddases [11]. In the case of RSV infection, two clinical studies have measured systemic serum or plasma levels of these molecules in peripheral blood and one study measured a panel of CAMs on neutrophils in peripheral blood and lung aspirates. Lai et al. measured sICAM-1 in sera from 10 healthy controls and 47 bronchiolitis patients at admission, and 25 patients at discharge [13]. The mean serum level of sICAM-1 in bronchiolitis patients was significantly higher than in the healthy control infants. However, improvement of the clinical severity score did not correlate with the change of sICAM-1 levels over time. Next, in a study by Øymar et al. serum levels of sICAM, sVCAM-1, sE-selectin, sL-selectin and sP-selectin, which are CAMs that are differentially expressed on leukocytes and endothelial cells, were analysed in 22 children needing hospitalization for their first episode of bronchiolitis [15]. The results showed a significantly higher level of sVCAM-1 and sL-selectin in serum of PCR-proven RSV-infected patients when compared to controls. There was no difference between the levels of CAMs in children with and without PCR-proven RSV bronchiolitis. Also, there was no information regarding the severity of bronchiolitis cases, so that appropriate correlations of CAMs and outcomes could not be made. Nonetheless, the limited number of studies discussed here found increased levels of soluble CAMs in RSV LRTI. These levels probably correlate with upregulation and release of these CAMs on the pulmonary endothelial cell and may represent shedding after recruitment, adhesion, and transmigration of neutrophils, which mechanism will be discussed below.

2.2. Evidence of neutrophil activation and transmigration in RSV infection

2.2.1. Neutrophil activation and transmigration in inflammation and infection

Neutrophils are the most abundant type of leukocytes in the peripheral blood. Inflammatory activation, such as stimulation with granulocyte colony stimulating factor (G-CSF) or interleukin-8 (IL-8), drives their release from the bone marrow and the appearance of specific neutrophil subsets, such as young or 'band' neutrophils, in the peripheral blood circulation [22]. To execute their effector functions, neutrophils migrate from the peripheral blood into the tissues. The underlying principles of transmigration have been reviewed extensively elsewhere [12,22–25]. In brief, circulating neutrophils first tether and role on the endothelium, mediated by E- and P-selectin on the endothelium and L-selectin on neutrophils and their respective glycoprotein ligands. Then, neutrophils interact with chemoattractants on the endothelial cell surface, which triggers adhesion, mediated by integrins such as CD11b-CD18 (Mac-1), leukocyte-function antigen-1 (LFA-1), both binding ICAM-1, or very late antigen-4 (VLA-4), binding VCAM-1, respectively. Neutrophils then migrate laterally over the endothelial surface before migrating across the endothelium into the tissues. Neutrophils migrate into the lung tissue mainly in the capillaries of the lung circulation and to a lesser extent from the postcapillary venules of the systemic circulation. Upon arrival in the tissues, neutrophils can degranulate (*e.g.*, release of neutrophil elastase), can produce myeloperoxidase (MPO) and reactive oxygen species (ROS), and can form neutrophil extracellular traps (NETs).

2.2.2. Neutrophil activation and transmigration during RSV infection

Inflammatory activation and transmigration of neutrophils has been widely studied in bacterial infection, but is poorly described for RSV infection. Some evidence is available from clinical and translational studies. First, a substantial number of clinical and post-mortem studies have shown that neutrophils are the main leukocytes found in the airways of infants with RSV bronchiolitis [7–9]. Furthermore, during RSV bronchiolitis an increased level of IL-8, a potent neutrophil chemoattractant, is present in airway secretions, which correlates positively with neutrophil counts, neutrophil elastase levels, and clinical severity scores [26–30].

Although these studies provide evidence for substantial neutrophil influx during RSV bronchiolitis, they do not address the route and mechanisms of transmigration of neutrophils from peripheral blood into the lungs. In a seminal study by Wang et al., an approach of flow cytometry and immunofluorescence was used to measure CAM and integrin expression on neutrophils in both peripheral blood and nasopharyngeal aspirates (NPA) of infants with RSV bronchiolitis [14]. The authors found lower expression of L-selectin, and a higher expression of CD11b, LFA-1 and ICAM-1, on neutrophils in NPA *versus* peripheral blood and in RSV-infected patients *versus* controls. Interestingly, influenza A virus infection of neutrophils *in vitro* had similar effects [31]. These results were repeated in a study by Halfhide et al., in which neutrophils in peripheral blood and in BAL from RSV-infected infants admitted to the ICU were studied using flow cytometry for the assessment of cell-surface marker expression and MPO production [32]. Neutrophils from RSV-infected infants had higher levels of CD11b in peripheral blood than controls. The finding that neutrophils in BAL of RSV-infected infants had even higher levels of CD11b, along with measurable levels of MPO, implies that neutrophil activation starts in the systemic circulation and continues during the course of migration into the alveolar space.

In a study by Lukens et al., several other aspects of neutrophil activation during RSV infection were documented [33]. Here, the total number of neutrophils in peripheral blood was monitored and, after immunofluorescent staining of neutrophils in whole blood with cell-surface marker CD16, neutrophils were phenotyped with flow cytometry. The authors found that the neutrophil numbers increased in the peripheral blood of young infants over the course of several days after admission to the intensive care unit due to RSV bronchiolitis, independent of bacterial co-infection. When compared to controls, RSV-infected infants had an increased fraction of neutrophil precursors (*i.e.*, myelocytes, metamyelocytes and banded neutrophils) with reduced expression of CD16 in peripheral blood, indicating recent recruitment from the bone marrow. These results were followed up in a recent study by Cortjens et al., in which four phenotypically distinct neutrophil subsets, based on CD16 and L-selectin expression as measured by flow cytometry, were identified in peripheral blood and BAL of young infants admitted with viral, predominantly RSV, infection [34]. The authors found high numbers of a subset of neutrophils, matching progenitor neutrophils, shortly after admission; these numbers increased over time, independent of bacterial co-infection. Although this specific subset of neutrophils was not retrieved in BAL of these infants, all other neutrophils in their BAL, which were mostly phenotypically mature, had reduced levels of cell surface L-selectin when compared to peripheral blood neutrophils. This may have been the result of active shedding of this molecule during extravasation, indicating neutrophil-endothelial interactions prior to appearance of the neutrophils in the alveolar space, which is congruent with the findings of Wang et al. and Halfhide et al. [14,32]. Only one study included experiments that

evaluate actual adhesion and transmigration of neutrophils across RSV-infected endothelial monolayers. In the *in vitro* study by Arnold et al., the authors found increased adhesion of neutrophils to RSV-infected endothelial monolayers [20]. When endothelial cells were cultured in a monolayer on a transwell membrane support, neutrophils migrated more avidly towards an interleukin-8 (IL-8) gradient, when endothelial cells were infected with RSV in a viral dose-dependent manner.

Last, in addition to the appearance of subsets of neutrophils with increased migratory properties in peripheral blood and lungs, several *in vitro* studies have suggested that RSV infection induces increased neutrophil activation effector functions, such as MPO and ROS production and NET formation [35,36]. Also, neutrophils may become infected directly by RSV, as suggested by the presence of RSV proteins and mRNA transcripts within neutrophils in peripheral blood and BAL from infants with severe RSV bronchiolitis [37]. A study in mice found that RSV RNA was present in peripheral blood and that RSV antigens were present in neutrophils in peripheral blood [38]. These results indicate that RSV infection of neutrophils may occur directly in the bloodstream. Interestingly, RSV blood RNA levels were associated with severity of disease.

3. Discussion

During RSV LRTI, both vascular endothelial cells and neutrophils in the systemic circulation can become activated and engage in interactions driven by neutrophil integrins Mac-1 and LFA-1 binding to endothelial ICAM-1 (Fig. 1). A pulmonary gradient of IL-8 serves as a potent chemoattractant for neutrophils and upon arrival in the alveolar space neutrophils further express integrins and execute effector functions. However, although some studies have found increased levels of soluble CAMs in RSV LRTI, these levels were not associated with severity of disease. Thus, our review raises important questions about the relevance of neutrophil-endothelial interactions in the pathophysiology of RSV LRTI and the use of its markers for clinical practice.

First, it has been suggested that extravasation of neutrophils into the airways may occur through mechanisms other than a well-orchestrated leukocyte adhesion cascade [12,22–24]. The narrow diameter of the pulmonary capillaries drives sequestration of neutrophils in the pulmonary vasculature. Immature neutrophils appear more rigid than their mature counterparts and bacterial infection and sepsis drives rigidity, which further increases neutrophil passage time and sequestration [22,25]. Extravasation occurs through integrin-ICAM-1 adhesion dependent or independent mechanisms depending on the stimulus, but the exact mechanism is not clear [25]. Moreover, in a rat model of LRTI with another viral infection, namely parainfluenza virus, the selective blocking of ICAM-1 reduced lung infiltration with neutrophils by 70 %, indicating that interactions of neutrophil integrins with endothelial ICAM-1 are the predominant mechanism for transmigration in this viral LRTI model [39]. Whatever the mechanism, neutrophils found in respiratory tracts of RSV-infected patients, must have passed the endothelium to enter the alveolar space. Thus, massive influx of neutrophils into the lungs occurring during severe RSV bronchiolitis is also a massive neutrophil *efflux* from the pulmonary vasculature.

The massive neutrophil efflux may cause collateral damage to the vascular endothelium and pulmonary epithelium. Evidence for the contribution of neutrophils in endothelial damage has been extensively reviewed for disease states such as sepsis, acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) [22,40–43]. Evidence from post mortem and animal (*e.g.*, mice and baboon) studies imply that pulmonary damage and edema may also be part of the pathophysiology of viral infections such as influenza, and of RSV LRTI [8,44,45]. For example, as a proxy of potential deleterious effects of neutrophils, levels of the proteolytic enzyme neutrophil elastase (NE) in the respiratory tract of young infants are correlated with severity of RSV-bronchiolitis [26,46]. Also, RSV particles and RSV F-protein can stimulate NET production from human neutrophils [35], which has been associated with both epithelial and endothelial damage in various disease states, such as sepsis and acute lung injury [47,48]. A particularly interesting finding in an *in vitro* study by Wang et al. is that neutrophil adhesion to human AECs was increased by RSV infection of these AECs and RSV infection was shown to cause damage and detachment of AECs, which was even further augmented by addition of neutrophils [49]. Thus, the positive correlation between neutrophil count and NE levels in lung aspirates with clinical outcomes, supports the assumption that the massive influx of neutrophils primarily aimed at controlling infection, also causes damage and consequently more symptoms [26,28,46].

While levels of soluble CAMs have been extensively evaluated in bacterial infection, there is scant data regarding RSV. Our review shows that levels of CAMs are raised during RSV LRTI and may be clinically relevant. However, whether CAM levels are associated with severity of RSV LRTI remains under debate. Available studies were not designed nor adequately powered to appropriately assess associations with clinical outcomes. Soluble CAM levels should be studied in large cohorts of patients with PCR-confirmed RSV-infection who are followed over time until resolution of infection or death. Assuming that there is a massive efflux of neutrophils in to the lungs with subsequent endothelial and epithelial damage, and that this is the basis for severe disease, it may well be possible that simultaneous measurements of markers of neutrophil and endothelial activation and integrity will be more predictive of severe disease than separate measurements alone. Particularly promising markers in this respect are the Angiopoietins Ang-1 and Ang-2 that bind to the endothelial Tie-2 receptor and regulate endothelial barrier integrity [50]. For example, increased plasma levels of Ang-2 are associated with the development and outcome of acute lung injury and ARDS and pulmonary vascular leak in adult critical illness [51–53], and plasma angiopoietin-2 levels were a prognostic marker for mortality in pediatric ARDS patients [54]. Whether Ang-2 plasma levels can also predict the clinical course of RSV LRTI needs to be determined.

In conclusion, there is evidence that activated neutrophils, activated vascular endothelium, and their interactions, are involved in the pathophysiology of RSV bronchiolitis. While these involvements appear intuitive, they have received little attention in the current literature, and deserve more attention in basic and clinical research. Such research may help to further unravel the pathophysiology of RSV bronchiolitis.

Authors' contributions

A.E. Juliana conceptualized the subject for the review, searched and analyzed the literature and wrote the manuscript. R. Zonneveld directly supervised the writing of the manuscript and provided critical feedback. All authors provided critical feedback, helped interpret data, and contributed to the outline of the manuscript.

Declaration of Competing Interests

The authors declare that they have no competing interests.

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