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REVIEW ARTICLE

Friend or Foe? The Role of the Host Cytoskeleton in Cellular Responses to Bacterial Pore Forming Toxins

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Abstract | Bacterial pore forming toxins (PFTs) are transmembrane proteins produced by pathogenic bacteria that increase infection severity in several instances. PFTs assemble into nano-sized pores on the host plasma membrane, making it permeable to ions and small molecules. As a response to PFTs, the host cell engages in cytoskeleton-mediated repair mechanisms to overcome the damage inflicted to its plasma membrane, and to maintain cellular homeostasis. However, PFTs themselves modulate the cytoskeleton in an attempt to escape host immune responses. Here, we review the changes that PFTs effect on the host cytoskeleton and how the host cell responds to this attack via cytoskeleton-associated pathways.

Introduction

Bacterial infections are one of the leading causes of mortality worldwide and include diseases such as cholera, pneumonia, and tuberculosis. Bacteria employ a variety of strategies to invade and colonize the host tissue[1]. A common strategy used by pathogenic bacteria to increase the severity and duration of infection is the formation of nano-sized pores on the membranes of host cells[2–4]. These pores comprise a special class of transmembrane proteins known as pore forming toxins (PFTs) which contribute to the virulence of pathogenic bacteria including *Listeria monocytogenes, Escherchia coli,* and *Streptococcus pneumoniae*[4].

PFTs are classified as α- or β-PFTs, based on the secondary structure of their transmembrane domain, i.e. an α-helix or a β-sheet, respectively[2,3]. Cholesterol-dependent cytolysins (CDCs) are β-PFTs which form large β-barrel, cholesterol-dependent pores. The CDC family of PFTs has been widely studied and includes a number of members such as suilysin (SLY) from *Streptococcus suis*, pneumolysin (PLY) from *Streptococcus pneumoniae* and listeriolysin-O (LLO) from *L. monocytogenes*[5]. Several others PFTs, including the *E. coli* α-PFT, cytolysin A (ClyA), have an enhanced activity in presence of cholesterol[6]. The Repeats-in-Toxin (RTX) family of PFTs has characteristic glycine-aspartate rich amino acid repeats at the C-terminal which are necessary for secretion[7]. Till date, 128 families of bacterial and non-bacterial PFTs have been identified according to the Transporter Classification Database (TCDB: www.tcdb.org).

PFTs are produced in an inactive water-soluble form which binds to lipids, sugars, or protein receptors on the host cell membrane[2–4]. PFTs such as cytolysin A (ClyA) produced by *E. coli* are activated by membrane-binding, while others such as aerolysin from *Aeromonas hydrophila* need an additional proteolytic step for their activation[8,9]. Upon membrane-binding, the diffusion of PFTs reduces and leads to an increase in local concentration of PFTs. This increased local concentration results in the oligomerization of PFT subunits required for pore formation. A crucial step in the formation of a pore is the insertion of a transmembrane domain into the host cell membrane via a series of conformational changes[2].

The transmembrane domains of PFTs are typically buried within the structure of PFTs. As is the case in ClyA, oligomerization and insertion of

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the transmembrane domain can occur simultaneously, leading to a functional but partially formed pore, which eventually forms a complete pore complex upon further oligomerization[8]. In other instances such as in aerolysin, oligomerization leads to the formation of complete but non-functional pre-pore intermediates, which eventually insert their transmembrane domains into the cell membrane to form a functional pore[9]. The property of switching from a soluble inactive form to a membrane-integrated pore complex upon exposure to the host cell surface is a characteristic feature of PFTs[2]. These nano-sized pores alter the host cell membrane permeability, causing ion imbalance, which leads to cell death in some instances[4].

Upon exposure to PFTs, the host cell attempts to get rid of these pores or to minimize downstream effects via mechanisms such as membrane repair, cytoskeleton remodelling, activation of signalling pathways such as mitogen-activated protein kinase (MAPK) to aid cell survival, or programmed cell death[4,5,10–12]. Recent studies also provide evidence for a cell survival mechanism that relies on the shedding of exosomes containing the receptor for the α-toxin from methicillin resistant *Staphylococcus aureus*[13]. The host cell response scales with the concentration of PFTs, with the cell engaging in survival strategies upon exposure to lower concentrations of PFTs and in programmed cell death under high concentrations[14–16].

Cellular responses to PFTs are in part mediated by a dynamic network of proteins collectively known as the cytoskeleton. In healthy cells, the cytoskeleton mediates a variety of functional and morphological changes such as division, migration, adhesion, and maintenance of overall shape and size. Cytoskeletal filaments provide structural support and play a vital role in the distribution of cellular components. The four types of cytoskeletal proteins in eukaryotic cells are: microfilaments or actin filaments (F-actin), microtubules (MTs), intermediate filaments (IFs) and septins. Here, we focus on F-actin and MTs since these are the two cytoskeletal components that have been primarily studied in context of PFTs[17–22]. F-actin polymers are composed of monomeric globular actin (G-actin) that assemble in a right-handed helical manner. Actin is the most abundant cytoskeleton protein and the actin cortex beneath the plasma membrane is largely responsible for membrane tension. F-actin is reorganized by actin-nucleating and binding proteins to form lamellipodia and filopodia. F-actin, together with non-muscle myosin II, forms contractile stress fibres, which have approximately 20–30 actin bundles. These stress fibres are important in adhesion and migration of the cell. MTs are hollow, tube-like structures composed of α and β tubulin heterodimers. MTs emerge from the centrosome located near the nucleus in most eukaryotic cells.

The form and organization of the F-actin cytoskeleton is regulated by molecular switches, i.e., GTPases, such as Rho, Rac1, Cdc42, and the actin nucleators formins, and Arp2/3 complexes. The dynamic nature of polymerisation and depolymerisation of F-actin ensures that cells organise these proteins at short notice during normal cellular processes as well as in response to environmental cues. Therefore, these proteins become the target for bacteria such as *L. monocytogenes* which hijack F-actin machinery to invade, internalise, and exploit the host[23]. F-actin and MTs also serve as tracks for motor proteins to mediate the active transport of cargoes such as mitochondria, other organelles and vesicles inside the cells. Both F-actin and microtubules possess an inherent polarity in their filament structure, with a more dynamic plus end and a less dynamic minus end. Motor proteins hydrolyse ATP to convert its chemical energy to mechanical work and bring about cargo transport or to exert forces. The MT-based motor protein cytoplasmic dynein mediates transport towards MT minus ends while the kinesin family of motor proteins is engaged in plus-end directed transport of cellular cargo. Myosins are F-actin-based motor proteins that bring about cellular transport and are the primary motors involved in muscle contraction. F-actin and myosin II motors forms contractile actomyosin bundles which are the basis of cell migration and force generation.

In this review, we focus on how the cytoskeleton is perturbed upon exposure to bacterial PFTs and how the host cell responds to PFTs via the cytoskeleton.

PFTs Remodel the Host Cell Cytoskeleton to Increase Infectivity and Evade Cellular Response

Formation of stress fibres, lamellipodia and filopodia are early-stage effects of PFTs.

Bacterial toxins including PFTs are known to modulate the cytoskeleton of host cells for their egress and to spread infection[24–27]. PFTs elicit time-dependent as well as dose-dependent responses from the host cell[14–16]. Prolonged exposure to PFTs increases the proportion of lysed cells, and with time, a saturation level of lysed cells at a given concentration is reached. Exposure

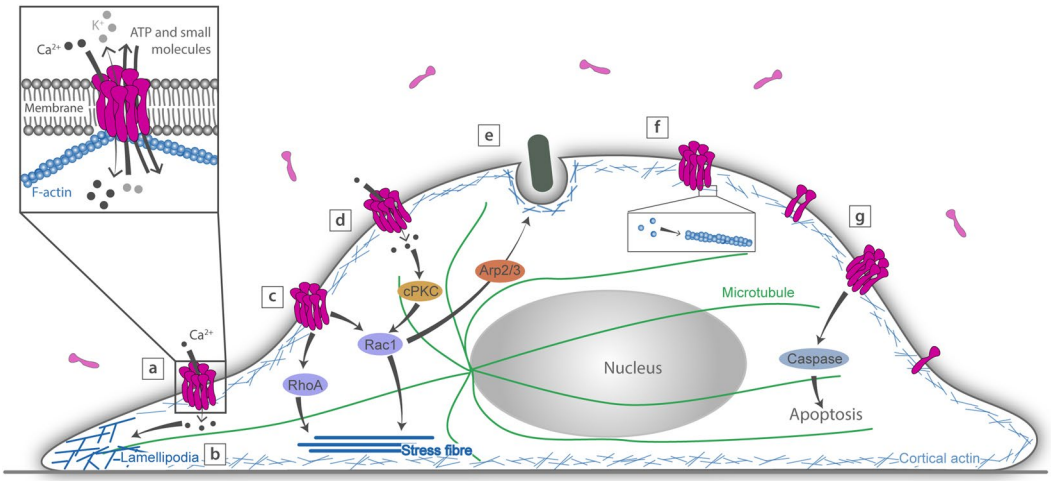

Figure 1: Modulation of the host cytoskeleton by bacterial PFTs. **a** Oligomerization of PFT subunits and formation of a pore on the host cell plasma membrane results in flux of calcium (inward) and potassium (outward) ions, ATPs and small molecules (both directions). **b** PFTs lead to the formation of cytoskeleton-based membrane protrusions such as lamellipodia. **c** The small GTPases Rac1 and RhoA are activated upon exposure of cells to PFTs to form stress fibres. **d** Calcium influx due to pore formation leads to the activation of Rac1 downstream of cPKC. **e** Rac1 remodels F-actin via Arp2/3 to effect internalisation of bacteria. **f** PFTs interact directly with F-actin and enhance the polymerization of G-actin to F-actin. **g** PFTs induce the activation of caspase signalling which results in programmed cell death.

of cells to lytic concentrations of PFTs leads to sudden rise in calcium levels (Fig. 1a), loss of cytosol through permanent permeabilization of plasma membrane and destruction of nuclear envelope, all of which lead to cell death[15,28]. On the other hand, exposure of cells to non-lytic concentrations results in the formation of membrane protrusions, active cytoskeleton remodelling and programmed cell death which might help increase the infection load. It has also been observed that a bi-component PFT YaxAB from *Yersinia enterocolotica* effects the time course and host pathology of *Yersinia* infection in mouse model[29].

Thermostable direct haemolysin (TDH), an atypical PFT from *Vibrio parahaemolyticus,* causes gastroenteritis in humans. Unlike other PFTs that oligomerize on the membrane, TDH forms tetrameric pore complexes prior to membrane binding[30]. Cytotoxic concentrations of TDH induce separation of cells from each other, loss of stress fibres, actin collapse, cell rounding, and membrane blebbing within 30 min of exposure[20,31]. Exposure of intestinal epithelial cells to non-cytotoxic concentrations of TDH leads to the formation of F-actin-and MT-based extrusions (Fig. 1b)[21]. So too, at sub-lytic concentrations, CDCs such as pneumolysin (PLY) from *Streptococcus pneumoniae* and suilysin (SLY) from *Streptococcus suis* induce the formation of stress fibres, filopodia, and lamellipodia[17,18]. CDC-induced stress fibres and lamellipodia start to appear within 4–8 min after toxin exposure and persist for as long as 60 min thereafter (Fig. 1b, c)[18]. As membrane protrusions such as lamellipodia are required for cell migration, the formation of these actin-based structures might aid the spread of the bacteria producing PFTs via regulation of host cell motility.

PFTs employ small GTPases to regulate the host cytoskeleton.

Formation of stress fibres, filopodia and lamellipodia are regulated by the small GTPases Rac1, RhoA, and Cdc42. PLY and SLY induce a threefold and fivefold increase in RhoA and Rac1 levels within 4 min of toxin exposure, respectively, without a concomitant increase in Cdc42 levels[17,18]. Treatment of cells with Rac1 and RhoA inhibitors prior to toxin exposure reduces the formation of these actin-based structures, implying a role for Rac1 and RhoA in the early F-actin remodelling steps[17,18]. So too, cells exposed to a mutant of SLY lacking haemolytic activity exhibit no activation of Rac1 or actin remodelling[17]. Actin remodelling is also effectively abrogated by preventing the first step in the pore-formation process—toxin binding to the membrane.

As CDCs bind to cholesterol on the host cell membrane, treatment of CDCs with cholesterol before exposure to cells renders these CDCs

incapable of binding to host membrane and results in the inhibition of actin remodelling[17,18]. Similarly, activation of small GTPases and formation of stress fibres, filopodia, and lamellipodia by CDCs is inhibited by removal of host cell cholesterol using methyl-β-cyclodextrin[17,18]. Interestingly, mutants of PLY with compromised pore-formation fail to induce retraction, cell shape and actin remodelling even at fivefold higher than sub-lytic concentration of wild type PLY[14,18]. A pre-pore locked LLO variant also inhibits Rac1 activation[32].

PFT-induced calcium influx remodels actin in a Rac1-dependent manner.

As a consequence of pore formation by PFTs, the host cell becomes permeable to ions, small molecules and proteins. The cellular calcium level spikes 10 min after PLY exposure, followed by a sudden decrease and sustained oscillations thereafter[15]. This oscillatory behaviour may be indicative of membrane repair. While PLY-induced lamellipodia formation does not depend on the calcium influx, activation of Rac1 and formation of filopodia reduce in a calcium-free buffer[18]. Similarly, LLO-induced Rac1 activation was significantly decreased in a calcium-free environment[32]. This might suggest that PFTs employ calcium-dependent as well as calcium-independent processes to remodel the host cytoskeleton. Pore formation by PFTs not only results in holes on the plasma membrane, but also internalisation of bacteria, redistribution of endoplasmic reticulum (ER), mitochondrial fission and alteration of host cell proteins levels, all of which eventually aid bacterial spread and egress[23,33–35]. For example, the CDC LLO from *L. monocytogenes* forms membrane pores which promote internalisation of the bacteria into the host cell by inducing endocytosis[23]. Other *Listeria* toxins such as invasins also mediate these processes, but LLO is sufficient to induce bacterial internalization in an actin-dependent manner. Specifically, this actin-mediated internalization requires calcium influx, which activates conventional protein kinase C (cPKC) signalling (Fig. 1d)[32]. An elevated cPKC level activates Rac1 GTPases, leading to Arp2/3 activation and F-actin remodelling needed for bacterial internalization (Fig. 1e)[23,32]. This suggests that decreased Rac1 levels in calcium free buffer upon PLY exposure and reduced actin remodelling thereafter might occur in a similar cPKC-dependent fashion[18].

PFTs bind and enhance oligomerization of actin.

PLY co-localises with F-actin within 4 min of toxin exposure followed by internalisation into the vesicles[19]. Furthermore, PLY has been observed to enhance the polymerisation of G-actin monomers resulting in increased F-actin to G-actin ratio (Fig. 1f)[19]. The direct interaction of F-actin with PLY was also seen in giant unilamellar vesicles (GUVs), which are micron-sized simple membrane models. GUVs loaded with PLY in a buffer containing Arp2/3 and monomeric G-actin displayed colocalization of PLY and F-actin through the lipid membrane[19]. Further, a pore-compromised mutant of PLY shows reduced interaction with F-actin[19]. This direct interaction of PLY with actin might be involved in calcium-independent actin remodelling by the PFT.

PFTs interfere with cell adhesion and lead to apoptosis.

Cell migration proceeds with the formation of lamellipodia and filopodia, and additionally requires crosstalk of the cytoskeleton with focal adhesion proteins. The RTX family PFT α-Haemolysin (HlyA) from uropathogenic *E. coli* (UPEC) disrupts MTs, F-actin, causes cell rounding and induces degradation of cell adhesion proteins including paxillin and β-catenin whereas a HlyA null mutant inhibits these changes[33]. However, the treatment of primary mouse astrocytes with sub-lytic concentrations of PLY results in increased vinculin focal adhesion and membrane retraction within 30 min of toxin exposure[14]. How PFTs regulates these focal adhesion proteins and their interaction with cytoskeleton remains elusive.

PFTs induce programmed cell death and activate other pathways such as cysteine-aspartate proteases (caspase) signalling (Fig. 1g)[15,16,31,33]. For instance, the α-toxin from *Staphylococcus aureus* induces apoptosis by activating caspase 2 at sub-lytic concentrations[16]. However, PLY-induced stress fibres are still observed in the presence of apoptosis inhibitory drugs[18]. This could be indicative of the temporal precedence of actin remodelling over apoptosis-induction upon PFT exposure.

The Host Cell Responds to a PFT Attack Via its Cytoskeleton

Cells form blebs to expel PFTs.

In response to PFTs, the host cell engages itself in membrane repair mechanisms such as clogging of pores, fusion of lipid vesicles, shedding of plasma membrane blebs containing PFTs, and induction of endocytosis for lysosomal degradation of PFTs[5,11,13,36–38]. These protection mechanisms all require extensive cytoskeleton

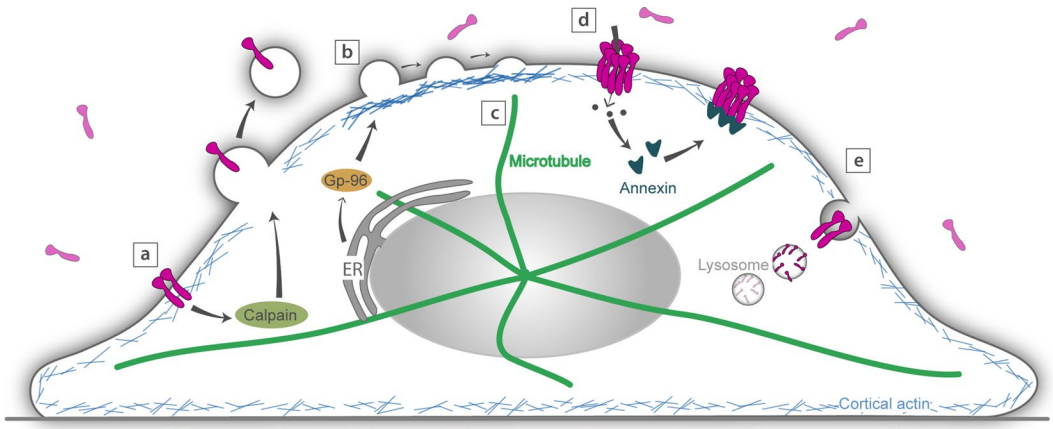

Figure 2: Host cell responses to PFTs via the cytoskeleton. **a** Calpain proteases degrade cortical actin to aid in the formation of blebs to expel PFTs. **b** ER redistribution and translocation of the ER protein Gp-96 to the plasma membrane regulate the assembly of cortical actomyosin bundles to maintain plasma membrane integrity. **c** MTs are stabilized in response to PFTs. **d** Annexins clog pores in a calcium-dependent manner. **e** Internalization and trafficking of vesicles containing pores formed in a RAB-dependent process help degrade PFTs in lysosomes.

remodelling. Early responses to the formation of pores involve disruption of actomyosin activity by the calcium-dependent cysteine proteases calpains, which degrade actin to allow the formation of blebs by reducing the plasma membrane tension (Fig. 2a). These blebs are later shed, thereby removing plasma membrane containing PFTs[22,35,39]. On the contrary, in conditions of high toxin load, the rise in intracellular calcium upon PFT exposure can also lead to cell death upon uncontrolled degradation of the cytoskeleton by calpains[10,40]. Reassembly of the actomyosin bundles upon shedding restores membrane integrity and thereby, cell homeostasis. In a process similar to formation of actomyosin bundles during cell migration, several PFTs including LLO, SLO, PFO, and AL induce the interaction of the ER heat shock protein Gp-96 and non-muscle myosin heavy chain IIA (NMHC IIA) at cortical plasma membrane and/or in blebs to form bundles to maintain the cell integrity (Fig. 2b)[22,35,39]. Pre-treatment of PFTs with cholesterol inhibits this distribution of Gp-96 and NMHC IIA to cortical bundles at plasma membrane blebbing sites[22,35,39]. Taken together, actin remodelling constitutes both short-term and long-term responses of host cells to PFT exposure and is necessary for the host cell survival at low toxin concentrations.

Cells exhibit stabilized MTs in response to PFT exposure.

PLY, which directly binds to F-actin, induces MT stabilization and bundling in neuronal as well as non-neuronal cells within 30 min of exposure (Fig. 2c)[19,41]. MT stabilization begins at the perinuclear region and spreads outwards with time[41].The stabilization of MTs upon PLY exposure is identical in calcium-containing or -free media, suggesting these PLY-induced MT changes are independent of the rise in intracellular calcium[31,41]. Similar to its effect on actin remodelling, depletion of or prior treatment of PFTs with cholesterol inhibits MT stabilization[18,41]. However, depolymerization or stabilization of F-actin has no bearing on PFT-induced MT dynamicity[21,41]. Alteration of microtubule stability is known to reorganize and remodel the actin cytoskeleton. Specifically, microtubule depolymerization activates GEF-H1 which acts via the RhoA pathway to increase F-actin polymerization and contractility[42]. The stabilization of MTs in the presence of PFTs could, therefore, counter the pro-bacterial actin remodelling steps discussed previously. In future, it will be interesting to explore the molecular underpinnings of the relationship between actin remodelling and MT stabilization observed upon PFT exposure.

Host cells employ annexins to clog pores on the membrane, or internalize pores to degrade them at lysosomes.

The calcium influx observed upon pore formation helps the host cell sense discontinuity in the plasma membrane and leads to the activation of several calcium-dependent processes, including redistribution of annexins to the plasma membrane and their subsequent accumulation at pores[10]. Annexin accumulation at the membrane helps clog the PFT-induced pore thus protecting the host cell (Fig. 2d)[39]. These clogged pores,

containing PFTs, annexins, myosins and calpains among others, are later shed[37]. Recent studies also reveal that the host cell sheds exosomes containing PFTs receptors to minimize damage[13]. In other instances, cells internalize PFTs bound to the plasma membrane for lysosomal degradation. This internalisation of PFTs is dependent on the small GTPases RABs, which are vesicle trafficking regulators (Fig. 2e)[36]. Several of these host cell strategies involve active transport of vesicles, primarily by the activity of kinesin and dynein motor proteins on the cytoskeleton. Future studies will help clarify the role of interaction amongst PFTs, the cytoskeleton, and the cellular transport machinery in the cell's strategy for mitigating the adverse effects brought about due to the toxin binding and eventual pore formation.

Future Perspectives

Recent advances in the field have revealed that pore-formation by PFTs affect the dynamics of mitochondria, ER, and various proteins including cytoskeleton[22,33–35]. However, in most instances, the precise molecular mechanism underlying these processes is yet to be discovered. These studies also indicate a complex role for the cytoskeleton in the formation of functional pores, flux of ions and loss of homeostasis, plasma membrane damage, and the induction of differential signalling cascades[14–19]. On the other hand, the host cell engages in protection mechanisms in response to PFTs[11,35–38]. The remodelling of the host cytoskeleton by PFTs as well as repair mechanisms effected via the cytoskeleton to overcome these challenges occur simultaneously, making it difficult to delineate PFT-mediated and cell-autonomous changes.

While direct interaction of PLY with F-actin has been documented[19], whether other PFTs bind and modulate F-actin in a similar fashion is yet to be uncovered. In addition, it is unknown if the other cytoskeleton components- MTs, IFs, and septins directly interact with PFTs, and how the cytoskeletal components talk to each other in response to toxin exposure. PFTs form pores with a range of diameters: the aerolysin family of PFTs forms pores of ~ 3 nm diameter, whereas PLY pores are ~ 26 nm in diameter[43,44]. This variety in pore sizes could also influence the selectivity and extent of the ion flux, differential activation of signalling cascades, and changes in cytoskeleton remodelling, and thereby varying durations for membrane repair following pore formation. For instance the repair of the large 30–50 nm pores formed by CDCs occurs in a timescale of seconds to minutes[38]. On the other hand, small pores such as those formed by haemolysin and aerolysin take hours for membrane repair[45]. Additionally, while the dose-dependent responses of the host cell to PFTs have been explored in some depth[14–16], a precisely tuned study exploring the time course of cellular changes with increasing concentrations of PFTs is yet to be performed.

Past research on the PFT-cytoskeleton axis pertains primarily to the CDC family, where cholesterol was found to enhance membrane binding by PFTs[2,5]. The role of cholesterol in mediating host responses in toxins such as ClyA where cholesterol hastens oligomerisation by stabilizing the promoter formation will also be interesting to explore[6]. Finally, the order of occurrence of the changes in the cytoskeleton effected by the PFTs, and of the responses mounted by the host cell are unknown and will likely form the focus of future studies in the field.

Publisher's Note

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Compliance with ethical standards

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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