

Ferritin Degradation by Pneumococcal HtrA, RadA and ClpP Serine Proteases : A Probable Way For Releasing and Acquisition Of Iron

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Purpose: Iron is a necessary element for the growth of bacteria; however, there are limited iron sources known for these microorganisms yet. Intracellular iron is stored as ferritin from, which releases iron in a gradual and controlled manner. The present study aimed to characterize ferritin-binding proteins (FBPs) of *Streptococcus pneumoniae*.

Material and Methods: *S. pneumoniae* species were cultured in BHI broth containing ferritin (1094 ng/mL) for 4h at 37°C. Ferritin level was measured using ELISA assay. Bacterial proteome was electrophoresed on SDS-PAGE and then transferred on PVDF nitrocellulose membrane. Afterward, the PVDF membranes were incubated with a ferritin solution. Identification of ferritin binding proteins was performed using anti-ferritin monoclonal antibody conjugated with HRP enzyme. Molecular docking was used to assess the interaction between pneumococcal proteases and FBPs applying phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor.

Results: No FBPs were identified in *S. pneumoniae* proteome. Moreover, ferritin levels have significantly ($p < 0.05$) decreased following the growth of *S. pneumoniae* in ferritin-rich BHI medium. Also, molecular docking showed that RadA protease, ClpP hydrolase, and HtrA protease can potentially interact with PMSF protease inhibitors. On the other hand, the addition of the PMSF to the culture of *S. pneumoniae* prevented the reduction of ferritin, which indicates a potential role of RadA, ClpP, and HtrA proteases in ferritin degradation.

Conclusion: Our results suggest that *S. pneumoniae* produces no FBPs and also cannot directly use ferritin as an iron source. However, ferritin may be degraded through a protease-mediated mode.

Keywords: *Streptococcus pneumoniae*, ferritin-binding proteins, proteome, iron

Introduction

Streptococcus pneumoniae is a pathogenic bacterium causing many infections such as meningitis, sinusitis, pneumonia, otitis media, and bacteremia.¹ Although *S. pneumoniae* presents as the normal flora within sinuses and nasopharyngeal tract in some individuals, it may also cause disease in people with compromised immune system such as young children and elderly. In fact, *S. pneumoniae* is considered as an important cause of meningitis, pneumonia, and sepsis in patients with HIV.

Iron is essential for the growth of bacteria, and particularly for pneumococci. In human beings, iron exists in combinations of glycoproteins such as ferritin, transferrin, and lactoferrin.² Therefore, iron is not easily available to bacteria, so they must recruit distinct strategies to earn this element.³⁻⁷

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Iron uptake mechanisms of *S. pneumoniae* that have not been thoroughly understood. Although some bacteria produce siderophore, this strategy has not been reported in pneumococci.⁸ In this regard, some studies have shown that ABC transporters (eg, Piu, Pia and Pit) constitute the main iron acquisition systems in pneumococci.⁹

Ferritin, as the major iron storing protein, plays a significant role in iron homeostasis by preserving up to 4,500 iron atoms in its structure.^{10,11} In addition, it comprises 24 units from two H and L chains and presents in the cytoplasm of cells and in the serum.¹² Iron is essential for *S. pneumoniae* to propagate within hosts; however, iron acquisition mechanisms of this bacterium are not fully understood yet. Therefore, in the present study, we aimed to characterize ferritin-binding proteins (FBPs) of *S. pneumoniae*.

Materials and Methods

Evaluation of Ferritin, Transferrin and Iron Levels in Pneumococcal Culture Bacterial Growth in BHI Medium Containing Human Serum

S. pneumoniae ATCC49619 was cultured in BHI broth for 24h at 37 ° C. Afterward, the culture medium was centrifuged at 9690 g for 3 min, and the pellet was re-cultured in BHI broth medium containing concentrated serum (1094 ng/mL ferritin, 135 µg/mL transferrin, and 88 µg/mL iron). Subsequently, the culture was further incubated in the candle jar for 4 hours at 37 ° C and finally centrifuged for 3 minutes at 9690 g. The supernatant was then separated to determine the levels of ferritin, transferrin, and iron using enzyme-linked immunosorbent assay (ELISA), immunoturbidometric, and photometric assays, respectively. The protocols were conducted in terms of the manufacturer's instructions (Pishtazteb & Pars Azmoon, Iran).

Evaluation of FBPs

Purification of Proteins by the Acetone and Ammonium Sulfate Methods

In this study, acetone was used to precipitate pneumococcal proteins in the culture supernatant.¹³ The following steps were performed to precipitate proteins:

1. The growth medium supernatant and cold acetone (−20 ° C) (the ratio of 1 to 4) were mixed into a falcon. After mixing, the falcon was incubated for 60 minutes at −20 ° C.

2. The sample was centrifuged for 10 min at 13000 g at −4 ° C.
3. The supernatant was cautiously discarded, and the Falcon was kept for 30 minutes at room temperature until the acetone was completely vaporized.
4. Distilled water was added to the falcon, and after 10 minutes of shaking, 2% SDS solution was added to the mixture.

The proteins into pellets of *S. pneumoniae* culture were also purified by ammonium sulfate method following cell lysis by sonication. Protein concentration was then measured using Nanodrop, and the precipitated proteins were stored at −70 ° C.

Western Blotting

The purified proteins and pneumococcus pellet lysate were boiled in sample buffer and electrophoresed on 10% SDS-PAGE. Another sample mixed with 2ME-free sample buffer and without boiling, and subsequently transferred on polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA). The membrane was blocked by 3% blocking buffer (PBS-1X containing 3% nonfat skim milk and 0.05% Tween 20) overnight at 4 ° C and rinsed three times with washing buffer (PBS-1X containing 0.05% Tween 20). The PVDF membrane was incubated with ferritin (62 ng/mL) solution at room temperature for 3 h and washed three times with the washing buffer. After that, the membrane was incubated (25 ° C, 1 h) with diluted (1:1000) anti-ferritin antibody conjugated to Horseradish peroxidase (HRP) (Pishtazteb, Iran) and then rinsed three times with washing buffer. Finally, the membrane was treated with 3, 3-diaminobenzidine (DAB) solution (Sigma-Aldrich, USA) for ~3 min. Plasma sample was also used as a positive control.

The Activity of Pneumococcal Proteases Against Ferritin and Transferrin

S. pneumoniae was cultured in BHI broth for 24 h at 37 ° C. Subsequently, the bacterial suspension was then centrifuged at 9690 g for 3 min, and the supernatant discarded. Accordingly, the pellet was transferred into a sterile tube containing transferrin (135 µg/mL) and ferritin (1094 ng/mL), and the tube was then incubated for 4 hours at 37 ° C. Tube without bacterium culture (Serum and BHI (SBHI)) and another containing 1mM PMSF (SBHI+PMSF) were also used as controls 1 and 2, respectively. After incubation, the tubes were centrifuged at 9690 g for 3 min. Finally, the concentrations of ferritin,

transferrin, and iron were measured using protocols mentioned earlier in section 1. All of the tests were repeated five times.

Docking of Pneumococcal Proteases with PMSF

A list of pneumococcal proteases was first obtained from the protein data bank (PDB) database and imported into Molegro virtual docker software (MVD 2013.6.0). Protease cavities were detected based on expanded Van der Waals on molecule surface and the following settings; minimum cavity volume: 10, maximum cavity volume: 10000, probe size: 1.20, max number of ray hits: 16, min number of hits: 12 and grid resolution: 0.80.

PMSF as a ligand was then imported into MVD software and molecular docking with tested proteases performed under the following parameters:

1. Scoring function: Score: MolDock, Resolution Grade: 0.30A
2. Binding site properties: radius 15, X axis (-1.95), Y axis (-0.97), and Z axis (-44.6).
3. Search algorithm: MolDock SE

All of the predicted poses were separately analyzed for hydrogen bond interaction, bond length (Å) and other types of interactions between protease and PMSF.

Statistical Analysis

Statistical analysis was performed using SPSS V22.0 software. Differences in values of Iron, transferrin, and ferritin between test and control groups were analyzed by one-way analysis of variance (ANOVA). Also, a P value of <0.05

was considered as statistically significant. All the graphs were drawn using GraphPad Prism 6.0 (San Diego, Calif.)

Results

Changes of Ferritin and Transferrin Levels Following Growth of *S. pneumoniae*

In the present study, the BHI medium enriched with serum (SBHI) containing ferritin (1094.71 ng/mL), transferrin (135 µg/dl), and iron (88 µg/dl) was used to grow *S. pneumoniae*. Notably, the levels of ferritin (P=0.001) and transferrin (P=0.047) have significantly decreased after a 4-hour growth of *S. pneumoniae*. On the other hand, free iron concentration has significantly increased after a 4-hour incubation (P= 0.006).

Exploring FBPs in Bacterial Proteome

Western blotting revealed no FBPs in the proteome of *S. pneumoniae*. Also, serum and plasma samples were used as positive controls (Figure 1).

Evaluation of Ferritin-Degrading Proteases

Considering the lack of FBPs in pneumococcus proteome and significant reductions in ferritin and transferrin levels in the culture medium of *S. pneumoniae*, there was a possibility of the degradation of ferritin and transferrin by pneumococcal proteases. Therefore, to test this hypothesis, ferritin and transferrin degradation tests were performed using PMSF serine protease inhibitors. *S. pneumoniae* was grown in SBHI medium containing ferritin, transferrin, and iron with the concentrations of 172.18 ng/mL, 182 µg/dl, and 91 µg/dl, respectively. In this regard, the obtained results showed that there were

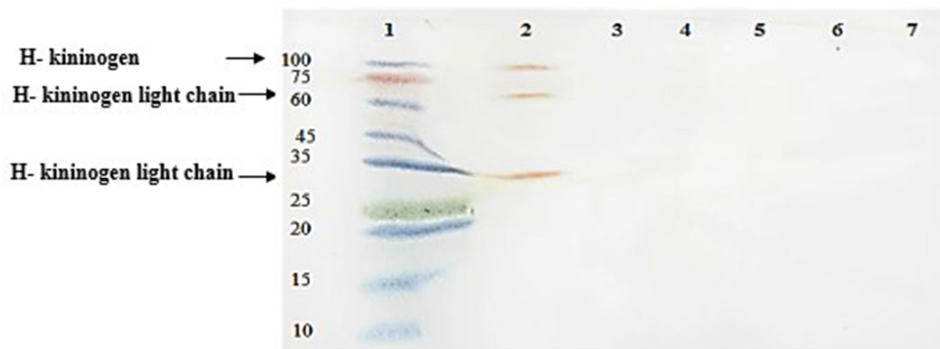


Figure 1 Western blot analysis of ferritin-binding proteins in *S. pneumoniae*. 1, protein marker; 2, plasma sample with 3 ferritin-binding proteins in ~30, 58 and 114 KDa ranges; 3, serum sample without any positive reaction; 4, a negative reaction with proteins concentrated from supernatant of *S.pneumoniae* culture; 5, a negative reaction with proteins from sediment of *S.pneumoniae* culture; 6, a negative reaction with reduced and denatured proteins concentrated from supernatant of *S.pneumoniae* culture; 7, a negative reaction with reduced and denatured proteins purified from sediment of *S.pneumoniae* culture.

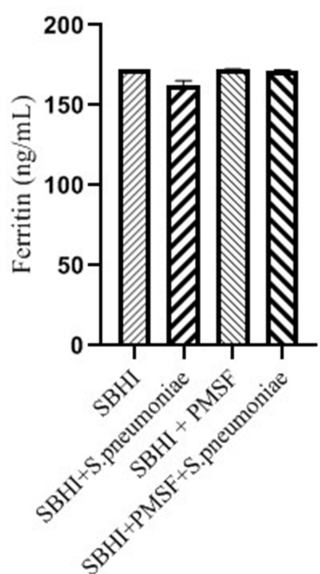


Figure 2 The levels of ferritin in the SBHI, SBHI + *S. pneumoniae*, SBHI + PMSF and SBHI + *S. pneumoniae* + PMSF groups.

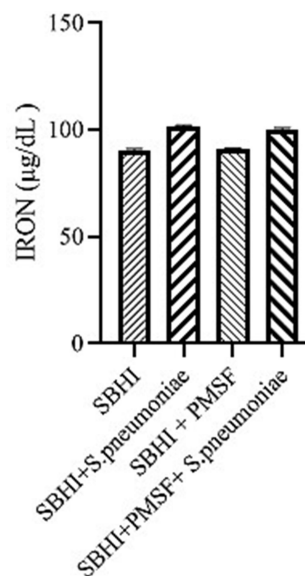


Figure 4 The levels of iron in the SBHI, SBHI + *S. pneumoniae*, SBHI + PMSF and SBHI + *S. pneumoniae* + PMSF groups.

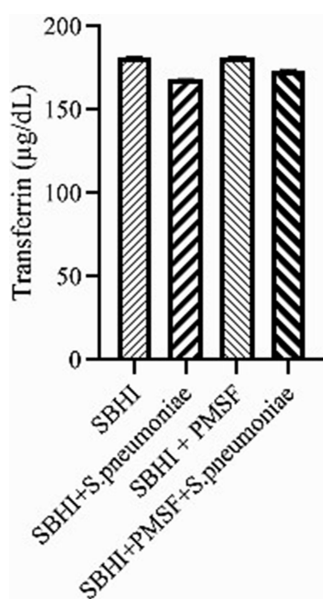


Figure 3 The levels of transferrin in the SBHI, SBHI + *S. pneumoniae*, SBHI + PMSF and SBHI + *S. pneumoniae* + PMSF groups.

significant reductions in the levels of ferritin compared to the control group1 (p: 0.000) and control group 2(p: 0.000), while there were no significant differences in the levels of ferritin between control groups1 and 2(p: 0.710). There were also significant reductions in the levels of transferrin compared to the control group1 (p: 0.000) and 2(p: 0.000). A significant difference was also observed in the levels of transferrin between the control group 1 and 2(p: 0.000), which show that transferrin has

been degraded by proteases other than those inhibited by PMSF (Figures 2–4).

Identification of PMSF Sensitive Proteases

Since PMSF is effective only on serine proteases, molecular docking (Molegro software) was used to confirm and also to identify PMSF sensitive proteases in the *S. pneumoniae* proteome.

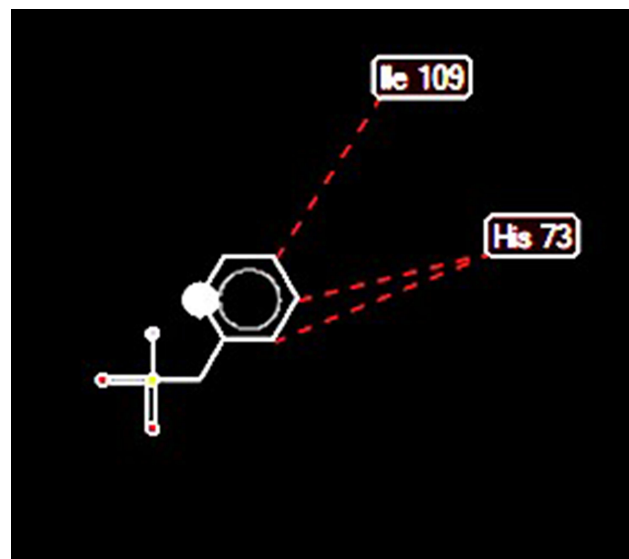


Figure 5 Interactions between PMSF and HtrA protease. Steric bonds between HtrA protease and PMSF at histidine 73 and isoleucine 109 positions were predicted.

Docking of HtrA Serine Protease with PMSF

Docking of HtrA serine protease (PDB accession number: 2L97) with PMSF indicated that PMSF was likely to bind to the protease at 5 positions. Moreover, high-grade protease analysis predicted two potential stearic bonds between PMSF and HtrA serine protease at His 73 and Ile 109 positions (Figure 5).

Docking of ClpP Serine Protease with PMSF

Docking of ClpP hydrolase (PDB accession number: 1Y7O) with PMSF indicated some possible interactions at 5 positions. In addition, high-grade protease analysis showed the interactions between two hydrogen atoms of PMSF and Arg 14 and Met 1 of the hydrolase (Figure 6).

Docking of RadA Protease with PMSF

Docking of the RadA (protease domain) hydrolase (PDB accession number: 5LKQ) with PMSF indicated some probable interactions at 5 positions. Moreover, high-grade protease analysis showed hydrogen bonds at the Gly 358 and Arg 330, as well as stearic interactions at the Gly 325, Lys 354, and Gly 357 positions (Figure 7).

Discussion

S. pneumoniae is known as an important cause of pneumonia, meningitis, and otitis media. Besides, it was shown that, the polysaccharide capsule and various virulence

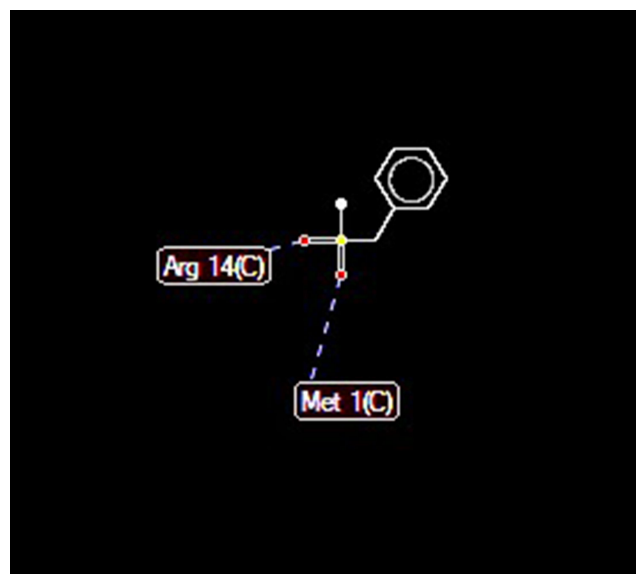


Figure 6 Docking between PMSF and ClpP protease indicated possible hydrogen bonds (length of 2.6 Å) at arginine 14 and methionine 1 positions.

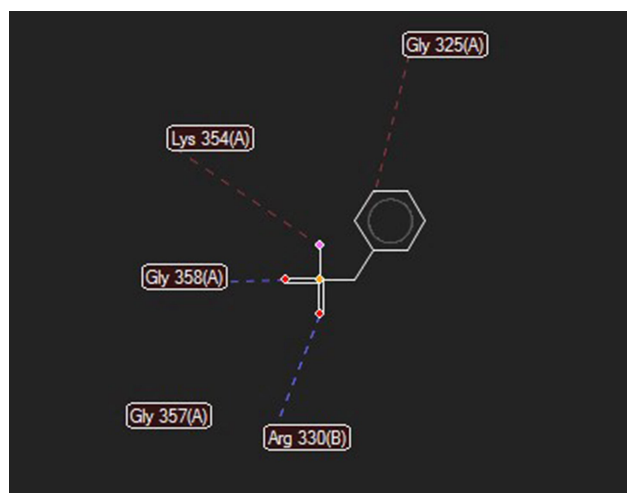


Figure 7 Docking between PMSF and RadA protease domain predicted hydrogen bonds at glycine 358 (length: 2.62737 Å) and arginine 330 (length: 3.11448 Å), and stearic bonds at glycine 325, lysine 354, and glycine 357 positions.

factors of *S. pneumoniae* play major roles in its pathogenesis and also facilitate its dissemination from the upper respiratory tract to the deeper regions of the lungs.

In the present study, we investigated the presence of FBPs in *S. pneumoniae* proteome. However, the results showed that *S. pneumoniae* had no FBPs. Moreover, it has been previously reported that *S. pneumoniae* lacks siderophores, and its iron uptake mechanisms are not well clear yet.¹³

Iron plays several important roles in the electron transport chain, energy metabolism, and many other biological functions.¹⁴ Accordingly, bacteria exploit different glycoproteins (eg, siderophores) to absorb iron from environmental sources and their hosts. Notably, many of these systems have been shown to play important roles in the pathogenesis of bacterial infections.¹⁵

Although hosts' tissues are relatively iron-rich, this iron is not easily accessible to bacteria, due to the actions of various iron chelating glycoproteins. Therefore, bacteria need efficient iron absorption systems to compete with the host's iron-chelating glycoproteins.¹⁶

The ability of pathogens in obtaining iron from transferrin, ferritin, hemoglobin, and other iron-containing proteins is considered as one of the most important factors ensuring their survival. Some pathogenic bacteria also acquire the host's iron either through the production of siderophores or through a direct contact with iron-containing proteins. Correspondingly, bacteria that lack these abilities are slowly growing and effectively eliminated by host's defense mechanisms.¹⁷

In human beings, iron is located within erythrocytes (ie, hemoglobin), ferritin, as well as in the serum and

secretions (eg, glycoproteins such as transferrin and lactoferrin).^{2,18}

Pneumococcal bacteria do not produce siderophore.¹⁹ In fact, the presence of a large capsule layer negates the presence of siderophore binding proteins on the bacteria surface. Therefore, siderophore binding proteins have no access to siderophore, which limits the applicability of these proteins for pneumococci. Lactoferrin is a transferrin-like protein present in secretions such as milk, tears, nasal secretions, saliva, bronchial mucosa, gastrointestinal fluid, and the bile. Also, iron-binding glycoproteins are associated with the pathogenesis of some bacterial species. In this regard, by producing lactoferrin-binding proteins; for example, *Neisseria* pathogenic strains use human lactoferrin as a source of iron.²⁰ Nevertheless, most of the non-pathogenic strains are unable to exploit iron-binding proteins.^{21,22}

In *S. pneumoniae*, PspA protein plays a key role in binding to lactoferrin at pneumococcal surface.²³ Moreover, lactoferrin is the only source of iron in epithelial secretions. Accordingly, it is essential for *S. pneumoniae* to bind to lactoferrin and to absorb iron via PspA for ensuring its proliferation and colonization. The expression of PspA has been shown to be associated with the reduced concentration of free iron in secretions.²⁴

Iron is also available in the structure of the “heme” within erythrocytes (ie, hemoglobin) and partially in the serum. In a study by Tai et al, it has been shown that pneumococcus produces a hemoglobin-binding protein (ie, PiaA) on the surface of its cytoplasmic membrane.¹⁹ Furthermore, Yang et al showed that the PiaA in a complex with ferrochrome siderophore is involved in iron transportation.²⁵ Moreover, in another study, Brown et al reported that ABC transporter-like proteins, may be involved in iron absorption by *S. pneumoniae*.¹³ In this regard, other possible iron transporters of *S. pneumoniae* have been identified as Pit, Pia, and Piu.²⁵

Ferritin is the main intracellular iron storage, which is also present in small amounts in the serum. Therefore, ferritin is considered as an important source of iron for microorganisms growing. In the present study, the results of Western blot analysis showed that *S. pneumoniae* had no FBPs. Actually, the thick polysaccharide capsule of *S. pneumoniae* can interfere with both binding of FBPs to ferritin and transporting of the complex into the bacterium. Despite this, ferritin concentration has significantly decreased in the culture medium of growing *S. pneumoniae*. Compared to the control group, ferritin level has significantly preserved after adding

a protease inhibitor (ie, PMSF) to the culture medium of *S. pneumoniae* ($p < 0.05$). Molecular docking results also showed that, pneumococcal proteases such as HtrA, ClpP, and RadA can be inhibited by the PMSF. Accordingly, these observations indicated that *S. pneumoniae* may recruit protease-dependent pathways to obtain iron. In fact, such pathways provide several effective strategies obviating the need for specific receptors and transporters. In addition, iron is a necessary element for the growth of bacteria; however, there are limited iron sources known for these microorganisms yet. Intracellular iron is stored as ferritin from, which releases iron in a gradual and controlled manner. The present study aimed to characterize ferritin-binding proteins (FBPs) of *S. pneumoniae*.

Conclusion

In the present study, we observed that *S. pneumoniae* had no FBPs, so it is unable to use ferritin as a direct source of iron. However, reduction of ferritin concentration in the culture medium of *S. pneumoniae* growing suggested FBPs-independent mechanisms for iron absorption. Moreover, preservation of ferritin level after using the PMSF protease inhibitor suggested a protease-dependent pathway for degradation of ferritin, probably as a source of iron.

Abbreviations

FBPs, ferritin-binding proteins; ELISA, enzyme-linked immunosorbent assay; PVDF, polyvinylidene difluoride; PDP, protein data bank; PMSF, phenylmethylsulfonyl fluoride.

Data Sharing Statement

The data and materials used during the current study are available from the corresponding and first authors on reasonable request.

Ethics Approval

The ethical code for the present study was IR.ZUMS.REC.1397.117.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest for this work.

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