Role of PII proteins in nitrogen fixation control of *Herbaspirillum seropedicae* strain SmR1

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Abstract

**Background:** The PII protein family comprises homotrimeric proteins which act as transducers of the cellular nitrogen and carbon status in prokaryotes and plants. In *Herbaspirillum seropedicae*, two PII-like proteins (GlnB and GlnK), encoded by the genes *glnB* and *glnK*, were identified. The *glnB* gene is monocistronic and its expression is constitutive, while *glnK* is located in the *nlmAglnKamtB* operon and is expressed under nitrogen-limiting conditions.

**Results:** In order to determine the involvement of the *H. seropedicae glnB* and *glnK* gene products in nitrogen fixation, a series of mutant strains were constructed and characterized. The *glnK*-mutants were deficient in nitrogen fixation and they were complemented by plasmids expressing the GlnK protein or an N-truncated form of NifA. The nitrogenase post-translational control by ammonium was studied and the results showed that the *glnK* mutant is partially defective in nitrogenase inactivation upon addition of ammonium while the *glnB* mutant has a wild-type phenotype.

**Conclusions:** Our results indicate that GlnK is mainly responsible for NifA activity regulation and ammonium-dependent post-translational regulation of nitrogenase in *H. seropedicae*.

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**Background**

The PII family comprises homotrimeric proteins that have important roles in the control of the central metabolism in bacteria and plants, acting as transducers of the cellular nitrogen and carbon levels [1,2]. In many *Proteobacteria* studied there is a pair of PII proteins, usually called GlnB and GlnK, and their function is to sense the cellular levels of nitrogen, carbon and energy by binding the effectors 2-oxoglutarate, ATP and ADP [2,3]. These signals are then relayed to target proteins through conformational changes triggered by interaction with the effectors. The proteobacterial PII proteins also undergo a cycle of uridylylation/deuridylylation catalyzed by the bifunctional GlnD protein [1] in response to the intracellular levels of nitrogen. These conformational and covalent state changes stimulate or inhibit interactions of PII with different cellular protein targets involved in nitrogen and carbon metabolism [2].

PII proteins are key players in the regulation of nitrogen fixation in *Proteobacteria*. In *Klebsiella pneumoniae* and *Azotobacter vinelandii*, GlnK is required to regulate the activity of NifL, which inhibits NifA, the *nif* gene specific activator, under nitrogen-excess conditions [4-6]. In *Azospirillum brasilense* and *Rhodospirillum rubrum* GlnB is necessary for the activation of NifA under nitrogen-limiting conditions [7-9], whereas in *Rhodobacter capsulatus* both PII proteins are necessary for the NH₄⁺-dependent regulation of NifA activity [10]. In addition, PII proteins are also involved in the post-translational control of nitrogenase activity in *R. rubrum* [11] and in *A. brasilense* through interaction with DraT, DraG and AmtB [12].

*Herbaspirillum seropedicae* is a nitrogen-fixing *β-Proteobacterium* isolated from the rhizosphere and tissues of several plants, including economically important species [13]. In this organism two PII-like coding genes were identified, *glnB* and *glnK* [14,15]. The *glnB* gene is monocistronic and its expression is constitutive [14], whereas *glnK* is apparently co-transcribed with *amtB* and *orf1*, which encode for an ammonium transporter and a membrane associated protein of unknown function, respectively [15]. Recently *orf1* was named *nlmA*.
(nitrogen limitation membrane protein A) since its product was detected in membrane extracts of *H. seropedicae* grown under nitrogen-limitation conditions [16]. The expression of the *nlmAglnKamtB* operon is dramatically increased under nitrogen-limiting conditions and is dependent on NtrC [15]. As in other *Proteobacteria*, both PII proteins from *H. seropedicae* are targets of covalent modification by GlnD (uridylyltransferase/uridylyl removing enzyme) in response to the levels of ammonium ions [17].

**Results and Discussion**

To analyze the role of GlnK and GlnB in the control of nitrogen fixation in *H. seropedicae*, glnB (LNglnB) and glnK (LNglnK) insertional mutants and a glnK in-frame deletion mutant strain (LNglnKdel) were constructed and their phenotypes analyzed under different physiological conditions. These mutant strains were able to grow using nitrate as sole nitrogen source (data not shown).

The effect of *glnB* and *glnK* disruption on the NtrC-dependent expression of the *nlmAglnKamtB* operon [15] was determined using chromosomal *amtB::lacZ* transcriptional fusions of strains LNamtBlacZ, LNglnBamtBlacZ and LNglnKamtBlacZ. These strains were grown under N-limiting (5 mmol/L glutamate or 2 mmol/L NH4Cl) or N-excess (20 mmol/L NH4Cl) conditions and assayed for β-galactosidase activity. The LNamtBlacZ strain grown under N-limiting conditions showed β-galactosidase activity 21 times higher than in high ammonium (Table 1), confirming that *nlmAglnKamtB* is highly expressed under N-limiting conditions [15]. Strains LNglnKamtBlacZ and LNglnBamtBlacZ revealed a similar pattern of *amtB* expression, indicating that the mutation of either *glnK* or *glnB* does not affect *nlmAglnKamtB* expression. Since *nlmAglnKamtB* transcription is NtrC-dependent, these results suggest that GlnB and GlnK can substitute for each other in control of the NtrC/NtrB system in *H. seropedicae*. In agreement with this suggestion, *ntrC* [18] and *glnD* (unpublished results) mutants strains of *H. seropedicae* are unable to grow on nitrate, whereas the *glnB* and *glnK* mutant strains can use nitrate as sole nitrogen source.

In *Escherichia coli* both GlnB and GlnK are involved in the regulation of NtrC phosphorylation by NtrB, although GlnB is more effective [19]. Although several attempts were made, we failed to construct a double *glnB*-*glnK* mutant suggesting that an essential role is shared by these proteins in *H. seropedicae*.

The effect of *glnK* or *glnB* mutation on nitrogenase activity of *H. seropedicae* was determined in cultures grown in NH4+-free semi-solid NFbHP medium (Figure 1). Nitrogenase activity was reduced by approximately 95% in both *glnK* strains (LNglnKdel and LNglnK) indicating that GlnK is required for nitrogenase activity in *H. seropedicae*. On the other hand, the *glnB* strain (LNglnB) showed activity similar to that of the wild-type. These results contrast with those reported by Benelli et al [14] who constructed a *H. seropedicae* *glnB::Tn5-20B* mutant (strain B12-27) that was unable to fix nitrogen. Immunoblot assays did not detect GlnK in the B12-27 strain [Additional file 1: Supplemental Figure S1], suggesting that a secondary recombination event may have happened in this strain resulting in loss of GlnK not observed by Benelli et al [14].

The nitrogenase phenotype of the *glnK* mutants was complemented by pLNOGA (*nlmAglnKamtB*) and also partially restored (about 50%) by a plasmid expressing *glnB* under control of its own promoter (pACB210) suggesting that a higher copy number of *glnB* can substitute for *glnK* under N-limitation. The lower nitrogenase activity of the *glnK* strains could be due to lack of nif expression or inhibition of nitrogenase. We therefore analyzed the effect of the *glnK* mutation on the NtrC-dependent *nifA* promoter [20] and on the NifA-dependent *nifB* promoter of *H. seropedicae* [21] by using plasmids carrying *nifA::lacZ* (pRW1) or *nifB::lacZ* (pEMS140) fusions (Table 2). The β-galactosidase activity was the same in both wild-type (SmR1) and glnK (LNglnK) strains containing *nifA::lacZ*, supporting the view that GlnK is not strictly necessary for NtrC regulation in *H. seropedicae* in the presence of a functional *glnB* gene. On the other hand, expression of the *nifB::lacZ* fusion was reduced 10-fold in the *glnK* mutant compared to the wild-type, indicating that GlnK is

### Table 1 Effect of *glnB* and *glnK* mutations on *nlmAglnKamtB* expression

| Growth Conditions | LNamtBlacZ (SmR1, *amtB::lacZ*) | LNglnKamtBlacZ (ΔglnK, *amtB::lacZ*) | LNglnBamtBlacZ (glnB-Tc, *amtB::lacZ*) |
|-------------------|---------------------------------|---------------------------------------|---------------------------------------|
| 5 mmol/L glutamate | (2.5 ± 0.2) × 10^3              | (2.4 ± 0.2) × 10^3                     | (2.3 ± 0.2) × 10^3                     |
| 2 mmol/L NH4Cl    | (2.1 ± 0.1) × 10^3              | (2.29 ± 0.08) × 10^3                   | (2.2 ± 0.1) × 10^3                     |
| 20 mmol/L NH4Cl   | (1.1 ± 0.2) × 10^3              | (1.4 ± 0.4) × 10^3                     | (1.6 ± 0.3) × 10^3                     |

Indicated strains of *H. seropedicae* were grown in the presence of glutamate or NH4Cl. β-galactosidase activity was determined as described. Values are the mean of at least three independent experiments ± standard deviation.
required for nifB expression in H. seropedicae, even in the presence of wild type glnB. These results indicate that the lower nitrogenase activity in the glnK mutants was the result of lack of nif expression, most likely due to impaired NifA activity.

Previous results showed that the N-terminal domain of H. seropedicae NifA is required for controlling its activity in response to NH4+, and that an N-truncated form of NifA is transcriptionally active, but not responsive to NH4+ levels [22,23]. Thus, the nitrogenase activity was determined in the glnK mutants carrying pRAMM1 or pLNA, which respectively express NmIA-GlnK-AmtB, GlnB, ΔN-NifA and NifA were also evaluated. Data represent the average of at least three independent experiments and bars indicate the standard deviations.

Figure 1 Nitrogenase activity of H. seropedicae wild-type, glnB and glnK strains Nitrogenase activity was determined as described using strains SmR1 (wild-type), LNglnB (glnB::Tc), LNglnK (glnK::Km), LNglnKdel (glnK::Km) grown in semi-solid medium. The glnK mutants carrying plasmids pLNOGA, pACB210, pLNA, or pRAMM1, which respectively express NmIA-GlnK-AmtB, GlnB, ΔN-NifA and NifA were also evaluated. Values are averages of at least three independent experiments ± standard deviation.

Table 2 Promoter activity of nifA::lacZ and nifB::lacZ fusions in H. seropedicae wild-type (SmR1) and glnK mutant (LNglnK) strains

| Strains    | β-galactosidase Activity [mmol o-nitrophenol/ (min.mg protein)] |
|------------|---------------------------------------------------------------|
|            | Plasmids                                                      |
|            | none              | pPW452 (promoter-less lacZ vector) | prW1 (nifA::lacZ) | pEMS140 (nifB::lacZ) |
| SmR1       | (3 ± 1) × 10      | (6 ± 2) × 10                       | (7 ± 1) × 10    | (2.8 ± 0.1) × 10    |
| LNglnK     | (2.0 ± 0.7) × 10   | (4 ± 2) × 10                       | (6 ± 1) × 10    | (2.5 ± 0.3) × 10    |

H. seropedicae strains carrying the indicated plasmids were grown in NFbHP medium supplemented with 10 mmol/L of NH4Cl under air at 30°C. The cells were then centrifuged, resuspended in NFbHP (nitrogen-free) medium and de-repressed for 7 hours under 1.5% oxygen. β-galactosidase was determined as described. Values are averages of at least three independent experiments ± standard deviation.
that shown by the glnK mutant[15]. These results allow us to propose a model for the regulation of nitrogen fixation in H. seropedicae. Under N-limiting conditions, NtrC-dependent promoters are activated leading to expression of nifA and nlmA/glnK/amtB genes. The status of fixed nitrogen is signaled to NtrC via the uridylylation state of either GlnB or GlnK. Under a low ammonium and oxygen condition, NifA activates the expression of nif genes in a process which requires GlnK, most probably in an uridylylated form. Thus, under N-limiting conditions the nitrogenase complex is active, AmtB is associated with the membrane, NlmA is most probably in the periplasm and GlnK is mainly located in the cytoplasm. When ammonium is added, deuridylylated GlnK rapidly associates with the cell membrane by interacting with AmtB to form the GlnK-AmtB complex which, in turn, signals to nitrogenase to switch-off by a yet unknown process.

Conclusions

In summary, our results show that both GlnB and GlnK proteins can regulate NtrC-dependent promoters in H. seropedicae. Under physiological conditions, GlnK is required for NifA activity control. GlnK also controls the nitrogenase switch-off in response to NH4+ by a mechanism which most probably involves the formation of a membrane-bound GlnK-AmtB complex.

Methods

Plasmids, Bacterial strains and Growth conditions

The H. seropedicae and E. coli strains and plasmids used in this work are listed in Table 3. E. coli strains were grown routinely in Luria medium (Luria broth or Luria agar) [29] at 37°C. H. seropedicae was grown at 37°C in NFbHP medium [30] supplemented with NH4Cl (20 mmol/L) or the indicated nitrogen source. The concentrations of the antibiotics used were as follows: ampicillin (250 μg/mL), tetracycline (10 μg/mL), kanamycin (100 μg/mL for E. coli, 1 mg/mL for H. seropedicae), streptomycin (80 μg/mL) and chloramphenicol (30 μg/mL for E. coli, 100 μg/mL for H. seropedicae).

Enzyme assays

β-galactosidase activity was determined in cells carrying a lacZ fusion as described [31]. To study the amtB-lacZ-Km® chromosomal fusion expression, H. seropedicae strains carrying chromosomal transcriptional fusions were grown for 14 hours in NFbHP medium containing glutamate (5 mmol/L) or NH4Cl (2 mmol/L or 20 mmol/L), and assayed for β-galactosidase activity. To study the nifA and nifB expression, H. seropedicae strains carrying plasmid-borne transcriptional fusions nifA::lacZ or nifB::lacZ were grown for 14 hours in NFbHP medium containing NH4Cl (10 mmol/L) under
Table 3 *Herbaspirillum seropedicae* strains and plasmids

| Strains | Phenotype/genotype | Reference |
|---------|--------------------|-----------|
| **Herbaspirillum seropedicae** | | |
| SmR1 | Wild type, Nif+, SmR<sup>+</sup> | [38] |
| LNglnK | SmR1 containing glnK::sacB<sup>-</sup>-Km<sup>R</sup> | this work |
| LNglnKdel | SmR1 containing ΔglnK<sup>+</sup> | this work |
| LNglnB | SmR1 containing glnB::Tc<sup>R</sup> | this work |
| LNamtBlacZ | SmR1 containing amtB::lacZ-Km<sup>R</sup> | this work |
| LNglnKamtBlacZ | LNglnKdel containing ΔglnK<sup>-</sup>-Km<sup>R</sup> | this work |
| LNglnBamtBlacZ | LNglnB containing ΔglnB<sup>-</sup>-Km<sup>R</sup> | this work |
| B12-27 | SmR1 containing glnB<sup>-</sup>-Tn5<sup>-268</sup> | [14] |
| **Escherichia coli** | | |
| DH10B | Sm<sup>R</sup>, F<sup>+</sup> [pproA<sup>B</sup>- lacZΔM15] | Life Technologies |
| S17.1 | Sm<sup>R</sup>, Tra<sup>+</sup> pro thi recA hsdR<sup>+</sup> (RP4-2 kan<sup>Tn7</sup> tet<sup>+</sup>Mu) | [39] |

| Plasmids | Relevant characteristics | Reference |
|----------|-------------------------|-----------|
| pACB192 | 1.7 kb DNA fragment containing the glnB gene of *H. seropedicae* in pSUP202 | This work |
| pACB194 | glnB gene of *H. seropedicae* with a tetracycline resistance transposon EZ-TN™ < TET-1 > (Epitecture) in pSUP202 | this work |
| pACB210 | glnB gene of *H. seropedicae* in pLAFR3.18Cm | this work |
| pDK6 | Expression vector/ lacZ promoter, Km<sup>R</sup> | [37] |
| pDK6nifACT | *H. seropedicae* nifA deleted of 606 bp in the 5' coding region cloned into pDK6 carrying the nifA promoter | this work |
| pDK6nifA | nifA gene promoter region of *H. seropedicae* in pDK6 | this work |
| pEM5140 | nifB-lacZ transcriptional fusion of *H. seropedicae* in pPW452 | [21] |
| pEM5301 | 1.7 kb EcoRI fragment that contains the promoter region and part of the nifA gene of *H. seropedicae* in pTZ19R | [40] |
| pLAFR3.18Cm | Tc<sup>R</sup>, Km<sup>R</sup>, IncP cosmid with the pTZ18R cloning nest | [15] |
| pLNfAfA | Expresses ΔN-NifA of *H. seropedicae* with its own promoter in pLAFR3.18Cm | this work |
| pLNOGA | 5.1 kb fragment that contains the nlmA<sup>-</sup>-KmR operon of *H. seropedicae* in pLAFR3.18Cm | [15] |
| pLNglK | 0.9 kb BamHI/HindIII fragment that contains the 3' terminal of the nlmA gene, the complete glnK gene and 5' terminal of the amtB gene of *H. seropedicae* in pTZ18R | this work |
| pMH701 | Km<sup>R</sup>, contains a sacB-Km<sup>R</sup> cassette | [35] |
| pPW452 | Tc<sup>R</sup>, lacZ gene fusion | [41] |
| pRAM277 | contains *H. seropedicae* nifA deleted of 606 bp in the 5' end, encoding an N-truncated form of NifA deleted of its N-terminal domain and Q-linker | this work |
| pRAMM1 | nifA of *H. seropedicae* in pLAFR3.18Cm | this work |
| pRW1 | nifA-lacZ transcriptional fusion of *H. seropedicae* in pPW452 | [20] |
| pSUP202 | Ap<sup>R</sup>, Cm<sup>R</sup>, Tc<sup>R</sup>, Mob | [39] |
| pSUPFamtBlacZ | Central region of the amtB gene with a lacZ-Km<sup>R</sup> cassette insertion in pSUP202 | [15] |
| pSUPGlnK | 0.9 kb BamHI/HindIII fragment that contains the 3' terminal of the nlmA gene, the complete glnK gene and 5' terminal of the amtB gene of *H. seropedicae* in pLAFR3.18Cm | this work |
| pSUPGlnKdel | ΔglnK (192bp) gene of *H. seropedicae* in pSUP202 | this work |
| pSUPGlnKaslacB | contains ΔglnK and a sacB-Km<sup>R</sup> cassette (from pMH701) cloned into the vector pSUP202 | this work |
| pSUPGlnKsacB | 0.9 kb fragment spanning from the 3' end of nlmA to the 5' end of amtB with a sacB-Km<sup>R</sup> (from pMH701) inserted into the glnK gene | this work |
| pTZ19R | Ap<sup>R</sup>, lacZ<sup>F</sup> tET<sup>+</sup> | [42] |
| pUC18 | Ap<sup>R</sup>, lacZ<sup>F</sup>, F<sup>+</sup> | Invitrogen |
| pUCG08del | 0.8 kb DNA fragment that contains the 3' terminal of the nlmA gene, the complete glnK gene and the 5' terminal of the amtB gene of *H. seropedicae* in pUC18 | this work |

Protein concentration was determined by the Bradford method [32] using bovine serum albumin as standard.

Nitrogenase activity was determined using cells grown in semi-solid NFbHP medium containing glutamate (0.5 mmol/L). For nitrogenase switch-off/on assays cells were incubated in 3 mL of NFbHP medium (O.D.<sub>600</sub> = 0.2) and incubated in 25 mL flasks, at 30°C for 7 hours under 1.5% oxygen. The results are reported as nmol of o-nitrophenol (NP) produced per min per mg protein.
were grown in liquid NFbHP medium with glutamate (4 mmol/L) at 30°C and 120 rpm [28]. Nitrogenase activity was determined by acetylene reduction [33,34].

**Construction of the LNglkB mutant of *H. seropedicae***

Plasmid HS26-FP-00-000-021-E03 (Genopar consortium, http://www.genopar.org), which contains the *H. seropedicae* glnB gene in pUC18, was linearized with EcoRI and treated with T4DNA polymerase. It was then digested with HindIII to release a 1.7 kb fragment containing the glnB gene. This fragment was subcloned into the vector pSUP202 previously linearized with BamHI, treated with T4DNA polymerase and digested with HindIII to produce plasmid pACB192.

*In vitro* transposon mutagenesis of the glnB gene carried by plasmid pACB192 was performed using the EZ:TN †-<TET-1> Insertion Kit (Epicentre Technologies) following the manufacturer’s instructions. A plasmid containing the transposon insertion in the glnB coding region was selected and named pACB194. This plasmid was introduced by conjugation to *H. seropedicae* SmR1 using *E. coli* strain S17.1 as the donor. Recombinant colonies were selected for tetracycline resistance and screened for the loss of chloramphenicol resistance (vector marker). Southern blot of restriction enzyme digested genomic DNA was used to confirm the presence of the transposon in the glnB gene (data not shown). This *H. seropedicae* glnB-Tc<sup>R</sup> strain was named LNglkB.

**Construction of the LNglKn mutant of *H. seropedicae***

To clone the glnK gene, chromosomal DNA of *H. seropedicae* was amplified using the primers glnKD (5′-AACGCTTCGATCCGCTACCTCGGT-3′, BamHI restriction site is underlined) and glnKR (5′-GGACCTGTATCTAGTGTATCCGT-3′, Xhol restriction site is underlined) were used to amplify a 180 bp region upstream of the glnK gene and the first 180 bp of glnK. The amplified fragments were joined by the HindIIIsite of the *H. seropedicae* SmR1 by conjugation with *E. coli* strain S17.1 as the donor. Recombinant colonies were selected for kanamycin and chloramphenicol resistance. One mutant strain was selected, and grown overnight in liquid NFbHP medium supplemented with ammonium chloride (20 mmol/L) and 80 μg/mL streptomycin. One microliter of the culture was plated on solid NFbHP medium supplemented with 20 mmol/L NH₄Cl, 5% sucrose and 80 μg/mL streptomycin. Sucrose is toxic to bacteria containing the sacB gene in the chromosome, therefore only strains that lost the sacB-Km<sup>R</sup> cassette by a second homologous recombination event would grow. The selected strains were analyzed by PCR with the primers GlnKF1 (5′-TGGTCCAAGACCTTGACG3′) and GlnKR1 (5′-CATGCTCATAGAGTTCGCC3′) which were homologous to the glnK flanking 5′- and 3′- regions, confirming the deletion of the 192 bp glnK fragment (data not shown). This in-frame glnK strain (ΔglnK) was named LNglnKdel.

**Construction of plasmid pLNΔNifA***

An Eco47III/SacI DNA fragment containing the nifA gene promoter region of *H. seropedicae* was excised from the plasmid pEMS301[36] and sub-cloned into the SmaI/SacI-cut vector pDK6 [37], yielding plasmid pDK6npifA. An XbaI DNA fragment encoding for the central and C-terminal region of NifA protein (ΔN-NifA) of *H. seropedicae* was excised from the plasmid pRAM2T7 and sub-cloned into the XbaI-cut pDK6npifA, in the same orientation as the nifA promoter, yielding plasmid pDK6npifACT. Finally, a SacI/HindIII DNA fragment containing the nifA 5′-truncated gene was excised from pDK6npifACT and sub-cloned into pLAFR3.18Cm digested with SacI and HindIII. The
generated plasmid was named pLNΔNifA and encodes for the central and C-terminal domains of NifA under control of the nifA promoter.

Construction of the plasmid pACB210
A 1.7 kb EcoRI-HindIII fragment containing the glnB gene with its promoter region was excised from the plasmid HS10-MP-00-000-014-E08 (Genopar consortium, http://www.genopar.org), and sub-cloned into the vector pLAFR3.18 digested with EcoRI-HindIII to yield plasmid pACB210.

Construction of chromosomal amtB::lacZ transcriptional fusions
To construct amtB-lacZ transcriptional fusions, the suicide plasmid pSUPamtBClacZ was introduced by conjugation, using E. coli strain SI7.1 as the donor, into H. seropedicae strains SmR1, LNglnKdel and LNglnB resulting in the strains LNamtBlacZ, LNglnKamtBlacZ and LNglnBamtBlacZ, respectively. Genomic DNA hybridization confirmed the presence of the cassette lacZ-Km in the amtB gene (data not shown).

Additional material

List of Abbreviations
ApR: ampicillin resistance; CmR: chloramphenicol resistance, KmR: kanamycin resistance; TcR: tetracycline resistance; SmR: streptomycin resistance.

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Authors' contributions
LN constructed plasmids and H. seropedicae mutants, carried out physiological experiments and helped to draft the manuscript; ACB constructed plasmids and carried out immunassays; RAM constructed plasmids and designed some of the experiments; LN, RAM and LUR helped to draft the manuscript; FOP, EMS, MBRS and LSC conceived the study, participated in its design and in writing the manuscript; LSC also supervised the study. All authors read and approved the final manuscript.

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