RANKL expression is differentially modulated by TLR2 and TLR4 signaling in fibroblasts and osteoblasts

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Abstract

Resident, non-immune cells express various pattern-recognition receptors and produce inflammatory cytokines in response to microbial antigens, during the innate immune response. Alveolar bone resorption is the hallmark of destructive periodontitis and it is caused by the host response to bacteria and their mediators present on the biofilm. The balance between the expression levels of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) is pivotal for osteoclast differentiation and activity and has been implicated in the progression of bone loss in periodontitis. To assess the contribution of resident cells to the bone resorption mediated by innate immune signaling, we stimulated fibroblasts and osteoblastic cells with LPS from *Escherichia coli* (TLR4 agonist), *Porphyromonas gingivalis* (TLR2 and -4 agonist), and interleukin-1 beta (as a control for cytokine signaling through Toll/IL-1receptor domain) in time-response experiments. Expression of RANKL and OPG mRNA was studied by RT-PCR, whereas the production of RANKL protein and the activation of p38 MAPK and NF-kB signaling pathways were analyzed by western blot. We used biochemical inhibitors to assess the relative contribution of p38 MAPK and NF-kB signaling to the expression of RANKL and OPG induced by TLR2, -4 and IL1 β in these cells. Both p38 MAPK and NF-kB pathways were activated by these stimuli in fibroblasts and osteoblasts, but the kinetics of this activation varied in each cell type and with the nature of the stimulation. *E. coli* LPS was a stronger inducer of RANKL mRNA in fibroblasts, whereas LPS from *P. gingivalis* downregulated RANKL mRNA in periodontal ligament cells but increased its expression in osteoblasts. IL-1 β induced RANKL in both cell types and without a marked effect on OPG expression. p38 MAPK was more relevant than NF-kB for the expression of RANKL and OPG in these cell types.

Keywords: Lipopolysaccharide, *porphyromonas gingivalis*, cell signaling, RANKL, OPG

Introduction

The essential role of RANKL/RANK/OPG axis in osteoclast differentiation/activation in inflammatory bone diseases, including periodontal disease is widely acknowledged [1]. In the inflamed periodontal microenvironment, various cell types are capable of expressing RANKL, including periodontal ligament fibroblasts, leading to tissue destruction through activation of RANK on pre-osteoclasts [2]. In fact, increased expression of RANKL associated with reduced expression of OPG was observed in both animal models [3] and human periodontally-diseased sites [1]. Specifically in periodontal disease, bacterial lipopolysaccharide (LPS) of Gram-negative microorganisms has shown both *in vitro* [4] and *in vivo* [5] an important role in damage process by inducing the expression of various inflammatory

mediators including: IL-1, IL-6 and IL-8 [6], which can indirectly promote RANKL expression which ultimately result in alveolar bone loss that was initiated by Toll-like receptors (TLRs) signaling [7]. LPS from Gram-negative bacteria of the subgingival biofilm associated with periodontal disease activate both TLR2 and -4 [8,9]. We have previously shown that TLR4 stimulation in periodontal ligament fibroblasts result in increased RANKL expression, which is at least partially dependent on p38 MAPK signaling [10]. In the periodontal tissues, gingival fibroblasts (GF) [11], periodontal ligament fibroblasts (PDL) [12] and osteoblasts [13] were shown to express TLR2 and TLR4 constitutively, which indicates that these cells are responsive to bacterial LPS [6]. In this context, these cell types that are classically thought of as contributing to periodontal tissue homeostasis [14] can also

participate in bone resorption during microbial aggression through secretion of many immunoregulatory cytokines [15]. Some of the cytokines produced by these resident cell types, such as IL-6 [16], may not only act synergistically with the pathogen-associated molecular patterns in the activation of dendritic cells and modulation of the adaptive immune response, but can also have direct effects on tissue degradation and bone resorption. Other inflammatory mediators and cytokines produced by the resident cell types in response to TLR signaling may have primarily direct effects on the modulation of tissue degradation, including matrix metalloproteases, PGE2 and RANKL. Thus, the cytokine network associated with diseased periodontal tissues is highly complex, and it is currently thought that the cytokine profile ultimately determines disease activity [17]. This cytokine profile is the consequence of the activation of different signaling pathways by a multitude of external signals present in the periodontal microenvironment, resulting in a signaling network that modulates cytokine production. Considering the infectious nature of periodontal disease and the crucial role of bacterial LPS as a ubiquitous and chronically present microbial stimulus activating TLR2 and -4, we studied LPS-induced activation of signaling pathways that are especially relevant for expression of inflammatory genes. LPS stimulation may have a positive role in the modulation of osteoclastogenesis by inducing RANKL and/or inhibiting OPG expression or a negative role by reducing the expression of RANK or M-CSF receptor on osteoclast precursor cells [18]. In human gingival fibroblasts, LPS had a bone-protective effect by inducing the production of OPG [19]. In this study we present data on the differential regulation of RANKL and OPG gene expression after activation of TLR2, -4 and IL1R in fibroblasts and osteoblasts, providing insight into the relative contribution of p38 MAPK and NF- κ B signaling to the expression of these genes in each cell type.

Material and methods

Cells and materials

Mouse periodontal ligament fibroblasts (mPDL), immortalized with simian virus 40 large T antigen, were originally obtained from Dr Martha J. Somerman (NIH/NIDCR, Bethesda, MD, USA). These cells were previously characterized for the expression of genes normally expressed by primary periodontal ligament cells, including bone sialoprotein, osteopontin, osteocalcin and type I collagen. Osteoblastic rat osteosarcoma cells ROS 17/2.8 (ROS) were obtained from Dr. Laurie McCauley (University of Michigan, Ann Arbor, MI, USA). Osteoblast phenotypic mRNAs, including bone sialoprotein and osteocalcin, were routinely assayed to verify osteoblastic phenotype expression in these cells. Unless noted otherwise, all tissue culture reagents were obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA). Lipopolysaccharide from *Escherichia coli* (serotype O55:B5) was purchased from Sigma-Aldrich (St Louis, MO, USA) and *Porphyromonas gingivalis* lipopolysaccharide was purchased from Invivogen (San Diego, CA, USA). Biochemical inhibitors of

p38 MAPK (SB203580) and NF- κ B (Bay-110782) were purchased from Sigma-Aldrich (St Louis, MO, USA). Both *E. coli* and *P. gingivalis* lipopolysaccharide were diluted in RNase-free water to 5 mg/mL. Recombinant human Interleukin-1 β protein was from R&D systems (Minneapolis, MN, USA) and was diluted in PBS containing 0.1% BSA. All primer pairs were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Mouse RANKL monoclonal antibody was obtained from Imgenex (San Diego, California, USA). Phosphorylated p38 monoclonal antibody was from Cell Signaling (Danvers, MA, USA), whereas the monoclonal antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the polyclonal antibody for phosphorylated p50 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Stimulation

Both mPDL and Ros17/2.8 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 100 IU/mL of penicillin, 100 ug/mL of streptomycin and 10% heat-inactivated fetal bovine serum, and maintained in a humidified atmosphere at 37°C and 5% CO₂. After plating, the cells were left to attach overnight in complete growth medium, routinely de-induced in medium containing 0.3% FBS for 6 h and then stimulated with the indicated concentrations of the agonists and for the indicated periods. In the experiments assessing the role of p38 MAPK and NF- κ B on RANKL and OPG gene expression, 10 uM of either SB203580 or Bay-110782 were added to the cultures 30 min before the stimulation with the LPSs or IL-1 β . All experiments were performed in duplicate and repeated in an independent manner a minimum of three times.

Semi-quantitative RT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer's instructions. The quantity and purity of total RNA were determined on a Biomate 3 (Thermo Electron Corporation) spectrophotometer. Complementary DNA was synthesized by reverse transcription of 500 ng of total RNA using 2.5 μ M Oligo (dT) 12-18 primers and 1.25 U/ μ L Moloney murine leukemia virus reverse transcriptase in the presence of 3 mM MgCl₂, 2 mM dNTPs and 0.8 U/ μ L of RNase inhibitor, according to the manufacturer's protocol (Improm II–Promega). The PCR reaction was performed in a MyCycler (Bio-Rad) thermocycler using 2uL of the RT reaction product on a 25 uL total volume PCR reaction mix (GoTaq Flexi, Promega). It was used 100 pmol/ μ L of each gene's primers (50 pmol/ μ L of sense and antisense primers) for RANKL, OPG and GAPDH genes yielding products of 467, 140, 503 and 418 bp for RANKL (mouse-mPDL), RANKL (rat-ROS) OPG and GAPDH, respectively. The primer pair used for RANKL–mouse–(accession no.: NM011613) was: sense 5'-CAGCACTCACTGCTTTTATAGAATCC-3'; antisense 5'-AGCTGAAGATAGTCTGTAGGTACGC-3'; for RANKL–rat–(accession no.: NM057149) was: sense 5'-TCGGGTTCCCATAAAGTCAG-3', antisense 5'-CTGAAGCAAATGTTGGCGTA, for osteoprotegerin (accession no:

NM008764) was: sense 5'-TG TAGAGAGGATAAACGG-3'; antisense 5'-CTAGTTATATGCAGCTTAT-3'; and for GAPDH (accession no.: BC083065) was: sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGA-CACATTGGGGTAG-3'. Optimized cycling conditions used for RANKL (from mouse) and OPG were: initial denaturation at 95°C for 2 min and 30 cycles (35 cycles for OPG) of: 95°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 7 min in the presence of 2.5 mM MgCl₂, whereas for RANKL (from rat) the cycling conditions were as follows: initial denaturation at 95°C for 2 min and 40/36 cycles of: 95°C for 15 sec, 58°C for 30 sec, 72°C for 30 sec and a final extension step at 72°C for 7 min in the presence of 2.5 mM MgCl₂. For GAPDH, RT thermocycler conditions were as follows: initial denaturation at 95°C for 2 min and 25 cycles of: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in the presence of 1.5 mM MgCl₂. The PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5 µg/mL). The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100-GE Healthcare), using Image J 1.45s software (National Institute of Health, USA-http://imagej.nih.gov/ij). The density of the bands corresponding to RANKL and OPG mRNA in each sample was normalized to the amount of the housekeeping gene GAPDH and expressed as fold change over unstimulated control.

Western blot

A total of 10⁵ mouse periodontal ligament cells (as well as of ROS osteoblastic cells) were grown for 24h in each well of six-well plates, de-induced by incubation for 12h in culture medium containing 0.3% fetal bovine serum and stimulated with either *E. coli* LPS (100 ng/mL or 1 µg/mL), *P. gingivalis* LPS (1 µg/mL or 10 µg/mL) or IL-1 β (1 ng/mL or 10 ng/mL) for 10, 20, 60 and 120 min for assessing activation of NF- κ B and p38 MAPK, and also for 24h and 48h for RANKL protein expression. Whole cell lysates were prepared by scraping the cells of each well of six-well plates with 100 µl of sodium dodecyl sulfate sample buffer (62.5 mM Tris HCl buffer, pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) on ice, followed by centrifugation at 9,000 RPM at 4°C for 5 minutes. 40 µL of this content was heat-denatured at 95°C for 5 minutes and separated on 10% Tris-Cl polyacrylamide gels run at 100 V for 90 min and subsequently electro-transferred to 0.2 µm nitrocellulose membranes (300 mA constant current for 60 minutes). The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies. The presence of the primary antibodies was detected on radiographic film by using HRP-conjugated secondary antibodies and a chemiluminescence system (Lumi-Glo, Cell Signaling). The membranes were stripped off both primary and secondary antibodies by 10-minute washing in dH₂O (2x) followed by incubation in stripping buffer (100 mM

2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 10 minutes at room temperature and a final 10-minute wash in Tris-buffered saline containing 0.1% Tween-20. After stripping, the membranes were blocked as described previously and incubated with antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to verify equal loading of the wells. Digitalized images of the radiographic films were obtained in a gel documentation system (ImageQuant 100, GE Lifesciences) and densitometric measurements were obtained using ImageJ 1.45s software (NIH, Bethesda, MD, USA-http://imagej.nih.gov/ij).

Statistical analysis

Pairwise comparisons between experimental groups were performed using the t-test with Welch's correction for unequal variances. Comparison between fold changes on mRNA expression between lipopolysaccharide-stimulated and untreated cells was performed with the one-sample t-test. The significance level was set to 5% and all calculations were performed using PRISM 4 software (GraphPad, Inc., San Diego, CA, USA).

Results

Differential activation of NF- κ B and p38 MAPK by TLR2, -4 and IL1R

TLR4, TLR2 and IL1R signaling was induced by stimulating both osteoblastic and fibroblastic cells with LPS from *E. coli*, LPS from *P. gingivalis* and with rhIL-1 β , respectively. In periodontal ligament fibroblasts, activation of p38 MAPK was biphasic using either LPS or IL-1 β . However, TLR signaling resulted in delayed activation of p38 in comparison to IL-1R signaling (10 and 60 minutes for IL-1R versus 20 and 120 minutes for TLR signaling; **Figure 1A**). Interestingly, activation of p38 MAPK was more intense with the lower concentration of LPS (100 ng/mL), whereas the higher concentration of LPS (1 µg/mL) resulted in a time-dependent activation with less evident peaks. In osteoblastic cells, TLR2 signaling was a more potent activator of p38 and NF- κ B than TLR4 signaling. Stimulation of IL-1R in these cells was of intermediary potency in comparison to TLR2 and TLR4, but similarly to the fibroblasts resulted in more rapid and transitory activation, in particular of NF- κ B (**Figure 1B**).

RANKL and OPG mRNA expression

Activation of TLR4 signaling in periodontal ligament fibroblasts resulted in a concentration-dependent increase on RANKL mRNA expression in later experimental periods (**Figure 2A**), whereas OPG mRNA levels were not affected. In contrast, TLR2 signaling significantly inhibited RANKL mRNA expression in these cells (**Figure 2B**), returning to basal/constitutive levels only 24 hours after stimulation with *P. gingivalis* LPS. There was also a trend to decrease OPG mRNA expression with activation of TLR2 signaling, but this inhibition was not as marked as for RANKL. In IL-1 β -stimulated periodontal ligament fibroblasts

Figure 1. Representative image of three independent western blot experiments for nuclear factor-kappa B p50 (NF-κB) and p38 mitogen-activated protein kinase (MAPK) evaluation. Periodontal ligament cells (A) and osteoblastic rat cells (B) grown on six-well plates were de-induced for 12 h in culture medium containing 0.3% fetal bovine serum and then stimulated with lipopolysaccharide from *Escherichia coli* or *Porphyromonas gingivalis* (100 ng/mL and 1 μg/mL) or IL1β (10 ng/mL and 100 ng/mL) for 0 (control-C), 10, 30, 60 and 120 minutes. *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; IL-1b, interleukin-1 beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

a more rapid induction of RANKL mRNA occurred with both IL-1β concentrations (Figure 2C), followed by a decrease in later experimental periods (18 and 24h). A discrete and rapid increase of OPG mRNA expression was also observed in earlier experimental periods, particularly with the higher concentration of IL-1β.

In osteoblastic cells, TLR4 stimulation by *E. coli* LPS (Figure 3A) also resulted in a greater induction of RANKL mRNA, which was directly proportional to the concentration of LPS. In contrast to the periodontal ligament fibroblasts, induction of RANKL occurred at early experimental periods, returning to the basal/constitutive levels 24 hours after stimulation. Interestingly, in osteoblasts the induction of OPG mRNA was inversely proportional to the concentration of *E. coli* LPS. Contrary to the inhibitory effect in periodontal ligament fibroblasts, activation of TLR2 by *P. gingivalis* LPS in osteoblastic cells increased RANKL mRNA expression, with approximately the same potency as IL-1b (Figure 3B). OPG mRNA levels fluctuated over time, but there was a trend of increased expression, especially after stimulation with *E. coli* LPS and IL-1 β.

RANKL protein production

Regulation of RANKL at the protein level supported *E. coli* LPS

as an inducer of RANKL, and this expression was sustained for 48h after the stimulation. Consistently with the inhibitory effect on mRNA expression, *P. gingivalis* LPS failed to induce RANKL protein at 24h; however, a slight but significant increase was observed after 48h. In contrast to the regulation of mRNA, IL-1 β was a more potent inducer of RANKL protein expression than *E. coli* LPS; however IL-1b-mediated induction of RANKL protein was observed only at 24h, with a return to the basal levels of expression at 48 hours (Figure 4A). In another contrast with the periodontal ligament fibroblasts, *E. coli* LPS was a more potent inducer of RANKL at the protein level in osteoblasts, and this induction was sustained for 48h after the stimulation. IL-1b and *P. gingivalis* LPS resulted in slight, albeit significant, increases on RANKL protein expression in osteoblastic cells (Figure 4B).

Utilization of p38 MAPK and NF-κB pathways by fibroblasts and osteoblasts

Expression of RANKL mRNA induced by TLR2, -4 and IL1R was dependent primarily on p38 MAPK in fibroblasts as indicated by the greater relative decreases on RANKL mRNA expression when the stimulations were performed with pre-treatment with 10 μM SB203580. Inhibiting NF-κB with 10 μM of Bay 11-

0782 attenuated RANKL expression after stimulation of TLR4 and IL1R, but not TLR2 in fibroblasts. On the other hand, in osteoblasts inhibition of p38 MAPK signaling reduced RANKL expression after stimulation of TLR2 and -4, but not after IL1R activation. Inhibition of NF- κ B reduced TLR4-induced RANKL expression, but it was associated with an increase on RANKL expression induced by TLR2 activation in these cells.

The discrete regulation of OPG mRNA was partly dependent on both p38 MAPK and NF- κ B activation in both fibroblasts and osteoblasts and for all stimuli, except for the slight increase associated with the activation of TLR2 in osteoblasts that was independent of NF- κ B (Figures 5A and 5B).

Discussion

In the periodontal disease microenvironment, the ubiquitous presence of various MAMPs and the widespread expression of PRRs by immune and non-immune cell types suggest that

innate immune signaling in 'non-professional'/resident cell types play a role modulating the host response. Importantly, cell type and differentiation state can influence both the expression of PRR, and also the signaling pathways activated by the same MAMPs [20].

Our research group has previously shown that p38 MAPK and its upstream activators MKK3 and MKK6 play an important role on lipopolysaccharide-induced RANKL expression by periodontal ligament cells [10], and in this study, we report that signaling through TLR2, TLR4 and IL1R results in differential modulation of RANKL gene expression. Moreover, a biphasic activation of p38 MAPK with delayed kinetics is associated with TLR signaling. The delayed activation of p38 MAPK upon TLR4 signaling is associated with a delayed induction of RANKL mRNA in comparison to IL1R signaling. Moreover, TLR4-induced expression of RANKL mRNA in fibroblasts was primarily dependent on p38 MAPK activation. A previous study

suggested that RANKL and OPG expression in periodontal ligament cells was indirectly induced by the production of IL-1 β and TNF- α 6 hours after LPS stimulation [21]. Our data show that induction of RANKL mRNA occurred mostly 8 hours or more subsequent to stimulation, so we cannot rule out a possible autocrine/paracrine influence by early-response genes in our results. Consistent with this possibility, when stimulated with IL-1 β both periodontal ligament fibroblasts and osteoblasts showed a more rapid increase of RANKL

mRNA. However, at the protein level these different agonists showed varying potencies in each cell type. *E. coli* LPS potently induced RANKL protein in osteoblastic cells, whereas IL-1b was a stronger inducer in periodontal ligament fibroblasts. It is tempting to speculate on potentially different roles for these cell types in periodontal disease based on their differential response to the same agonists: osteoblasts were more responsive to TLR signaling and periodontal ligament cells more sensitive to inflammatory mediators, e.g., IL-1, that

may be produced in response to the initial bacterial challenge or in situation associated with mechanical stress such as orthodontic movement or traumatic occlusion. Activation of nuclear factor-kappa B (NF-κB) followed a similar pattern regardless of cell type, suggesting it is receptor-dependent with distinct results for TLR or IL1R stimulation. In both periodontal ligament fibroblasts and osteoblastic cells only IL1R signaling resulted in biphasic activation of NF-κB (especially with the lower concentration of IL-1b), whereas TLR2 and TLR4 signaling was associated with a more sustained activation over time. IL-1β signaling through IL1R may also follow an alternative pathway that is associated with the second peak of activation of NF-κB representing delayed NF-κB activation through TAK1,

which, together with TRAF6, is also an upstream activator of MAPKs [22]. Interestingly, regulation of RANKL and OPG mRNA induced by TLR2, -4 and IL1R was primarily dependent on p38 MAPK in both fibroblasts and osteoblasts; with NF- κ B activation, a major downstream signaling effector of TLRs and IL1R, having a more prominent role on IL1 β -induced RANKL mRNA expression by fibroblasts.

The inhibitory effect of on RANKL mRNA upon stimulation with *P. gingivalis* LPS is both unexpected and intriguing. It is possible that this effect may represent a cell-type specific effect, as this inhibition was not observed in osteoblastic cells stimulated with *P. gingivalis* LPS. Interestingly, downregulation of RANKL and increased OPG mRNA expression was already reported in cementoblasts stimulated with *P. gingivalis* LPS [23]. We did not find consistent changes of great magnitude on OPG mRNA expression on fibroblasts and osteoblasts, suggesting that the agonists used to activate TLR and IL1R signaling did not evoke a protective feedback mechanism to maintain bone homeostasis. The relatively minor changes in OPG expression further suggests that the ultimate outcome of bone turnover induced by these agonists might be determined by the changes on the expression of RANKL.

These results illustrate how gene regulation induced by the same agonists may vary depending on cell type, as well as the differential utilization of intracellular signaling pathways in these different cell types. Periodontal ligament fibroblasts have been shown to be relevant for bone formation in periodontal regenerative procedures and also to express RANKL in response to different interleukin-1, lipopolysaccharide, prostaglandin E2 and mechanical stress [24-26] by the activation of mitogen-associated protein kinases (MAPKs). In osteoblasts exposed to the whole bacteria *Porphyromonas gingivalis*, RANKL mRNA levels were dependent on activation of activator protein 1 (AP-1); but, surprisingly, this activation did not require p38, ERK MAPKs or the phosphoinositol-3-kinase (PI3K) pathway [27]. Recent information indicates that activation of TLR2 or of TLR4 in non-committed osteoclast precursor cells may inhibit RANKL-induced differentiation. This effect is attributed to antagonistic effects of NF- κ B activation induced by TLR signaling and NFAT-c1 activation induced by RANKL signaling [28].

NF- κ B and p38 MAPK are involved in the expression of various inflammatory mediators that play a role in the tissue degradation associated with periodontal diseases. We show that both LPSs and IL-1 β were able to activate these signaling pathways in periodontal ligament fibroblasts and osteoblastic cells and also modulate the expression of RANKL and, to a lesser extent, of OPG. Interestingly, regulation of these genes at the mRNA level was primarily dependent on p38 MAPK. The varying responses to TLR signaling by different cell types present in the periodontal tissues suggest different roles in the response to bacterial challenge. Understanding the potential role of different cell types and their activation mechanisms can contribute to the development of future strategies in

periodontal disease treatment based on interfering with signal transduction.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	FRML	SGA	MRG	JAC	CR
Research concept and design	--	--	--	--	✓
Collection and/or assembly of data	✓	✓	✓	--	--
Data analysis and interpretation	✓	--	--	✓	✓
Writing the article	--	✓	✓	--	✓
Critical revision of the article	--	--	--	✓	✓
Final approval of article	--	--	✓	--	✓
Statistical analysis	✓	--	--	--	--

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