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Short Communication

Plasma levels of Semaphorin 4D are decreased by adjuvant tamoxifen but not aromatase inhibitor therapy in breast cancer patients

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ABSTRACT

Background: Semaphorin 4D (Sema4D) is a glycoprotein that inhibits bone formation and has been associated with cancer progression and the occurrence of bone metastases. Recently, Sema4D expression has been linked to estrogen signaling in breast cancer. Endocrine therapies like tamoxifen and aromatase inhibitors (AI) are a standard therapeutic approach in hormone receptor positive breast cancers. Tamoxifen exerts ER-agonistic effects on bone, whereas AI negatively affect bone health by increasing resorption and fracture risk. The effect of endocrine therapies on circulating Sema4D levels in breast cancer patients has not been investigated yet.
Methods: We measured circulating Sema4D plasma levels at primary diagnosis and in a follow-up sample 12 months after surgery in a cohort of 46 pre- and postmenopausal women with primary estrogen receptor positive breast cancer receiving adjuvant tamoxifen or AI.
Results: The mean baseline levels ± SD for Sema4D were 441.6 ± 143.4 pmol/l. No significant differences in total plasma Sema4D were observed when stratifying the patients according to age, menopausal status, tumor subtype, nodal and hormone receptor status, or tumor size. However, Sema4D levels were significantly reduced by 28% ($p < 0.001$) in tamoxifen treated patients 12 months after surgery, whereas no alteration was observed in patients treated with AI.
Conclusion: This finding potentially represents an additional mechanism of the bone-protective properties of tamoxifen and further emphasizes a link between Sema4D and estrogen receptor signaling.

1. Introduction

Semaphorin 4D (Sema4D) is a transmembrane homodimer glycoprotein and member of the Semaphorin family which consists of more than 20 genes. Originally, Sema4D was identified as a major immune regulator [1]. In addition, several studies revealed that Sema4D is associated with bone remodeling and cancer progression. The protein is abundantly expressed by differentiating osteoclasts, binds its receptor Plexin-B1 on osteoblasts and inhibits osteoblastic function by suppressing insulin-like growth factor-1 (IGF-1). In mice, a global knockout of Sema4D leads to an increased bone volume and the antibody-mediated neutralization of Sema4D in a osteoblast-osteoclast co-culture increases osteoblastic bone formation [2]. In addition osteoclasts with knocked-out Sema4D display a significant bone resorption defect [3]. Interestingly, elevated levels of soluble Sema4D are found in the serum and bone marrow of multiple myeloma patients, a malignant disease that is accompanied by the presence of osteolytic bone lesions [4]. In ovarian cancer, Sema4D is upregulated in human cell lines. In these cells, the ERα was shown to accelerate tumor cell proliferation at least partially by positive regulation of Sema4D. In addition, Sema4D is increased in the serum of affected patients with ovarian cancer [5]. In addition, Plexin-B1 is overexpressed in primary prostate cancer tissue [6].

Abbreviations: AI, aromatase inhibitors; ER, estrogen receptor; Sema4D, Semaphorin 4D

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Sema4D signaling is involved in tumor angiogenesis as Plexin-B1 is highly expressed in endothelial cells [7]. A supportive role of Sema4D in tumor progression was further revealed by showing that its neutralization promotes immune cell infiltration into the tumor microenvironment and shifts the immune response towards a pronounced pro-inflammatory and antitumor milieu [8]. In breast cancer, the role of Sema4D signaling appears increasingly complex. While the knock-out of Sema4D in breast cancer cell lines suppressed xenograft growth and angiogenesis [9] and decreased the occurrence of bone metastases [10], a reduced expression of Sema4D has also been described to be associated with a poor prognosis [11]. An increased expression of Sema4D has been described in estrogen receptor positive breast tumors and Sema4D was up-regulated in estrogen receptor (ER) positive breast cancer cell lines, following the exposure to ER agonists [11].

In this study we aimed to investigate i) whether baseline Sema4D levels differ among breast cancer patients with different clinical characteristics and ii) if the adjuvant treatment of breast cancer patients with tamoxifen or aromatase inhibitors (AI) affects the levels of circulating Sema4D.

2. Patients and methods

2.1. Patient population, patient characteristics and study design

In this study 46 consecutive patients with histologically confirmed early invasive breast cancer who underwent surgical treatment at the Department of Gynecology and Obstetrics at the University Hospital Dresden, Germany between 2013 and 2014 were included [12]. Patients had not received any prior treatment at the time of inclusion. Informed written consent was obtained from all patients. The study was approved by the Institutional Review Board (Dresden EK 236,082,012) and performed according to the declaration of Helsinki. Inclusion criteria were early disease with no sign of metastases and confirmed tumor expression of the ER. Patients with neoadjuvant treatment were excluded. Surgical procedures were breast conserving surgery or mastectomy. Adjuvant (anthracycline and taxane based) chemotherapy was given in 57% of cases with a higher risk of relapse. Radiation therapy was performed in 85% of the patients. Adjuvant endocrine therapy was performed in all patients with either tamoxifen (29/46; 63%) or an aromatase inhibitor (exemestane, letrozole, and anastrozole; 17/44; 37%)). We considered women perimenopausal when they were 40 years or older and were premenopausal before chemotherapy treatment but neither clearly pre- nor postmenopausal afterwards. Patients were treated according to standard guidelines (AGO 2013). Her2 positive breast cancer patients (6/46; 13%) were treated with trastuzumab.

2.2. Plasma sampling

Blood was drawn at primary diagnosis before any treatment, and one year after primary surgery. Nine ml blood were obtained from each patient with a EDTA S-Monovette® (Sarstedt AG & Co., Nuembrecht, Germany), directly stored at 4 °C and processed within 4 h to avoid blood cell lysis. Blood was fractionated by centrifugation for 8 min at 1800 × g at room temperature. Afterwards, the plasma fraction was removed and stored at −80 °C until analyses for Sema4D were performed.

2.3. Measurement of soluble Semaphorin 4D by ELISA

Human soluble Sema4D plasma concentrations were measured by ELISA (Biomedica, Vienna, Austria). Briefly, 10 µl of undiluted plasma were mixed with 100 µl of assay buffer on coated microtiter stripes. After 3 h at room temperature, wells were washed and conjugate was added to each well. After another hour, wells were washed again and 100 µl of substrate was added to each well. After 30 min of incubation, the stop solution was added and the absorbance was measured immediately at 450 nm with reference at 630 nm.

2.4. Statistics

For the comparison of Sema4D levels within the patient cohort, baseline levels were analyzed for the whole patient cohort and after stratifying the patients according to clinical parameters and treatment groups. Mean ± SD was calculated and compared between the groups using one-way ANOVA (Table 2). For the comparison of Sema4D time-course levels in the two treatment groups, data are presented as box-whisker blots of absolute values of Sema4D at two time points in both the AI and tamoxifen group and as absolute and relative differences of Sema4D levels 12 months after diagnosis compared to basal levels before surgery. Absolute and relative changes of Sema4D over time are additionally presented as median. Outliers were determined via Grubb´s test. Single group comparisons were performed by a Student's *t*-test and group analyses were performed using one-way analysis of variance (ANOVA) by GraphPad Prism 6.03 (GraphPad, La Jolla, CA, USA). P-values <0.05 were considered statistically significant. Final arrangement of the figures was performed using CorelDraw® X6 version 16.0.0.707.

3. Results

3.1. Patient cohort

The cohort consisted of 46 breast cancer patients as previously described [12]. Baseline patient characteristics at the time of primary diagnosis are shown in Table 1. The median age was 61 (range 26–85 years) and the median weight 75 kg (range 50–115 kg). The majority of patients (67%) were postmenopausal. The most predominant tumor type was Luminal A (71.7%), 15.2% were luminal B Her2 negative and 13% were classified as Luminal B Her2 positive. Only ER positive tumors were included. Of these, 93.5% displayed a concurrent expression of the progesterone receptor (PR). Tumors of 6 patients (13%) showed positive Her2 expression. The majority of the patients had T1 tumors (36/46, 78.3%), 6 patients had T2 tumors (13%), and 4 patients had T3/T4 tumors (8.7%). Of all patients, 71.7% (33/46) were lymph node negative. Patients received adjuvant therapy with either tamoxifen (29/46; 63%) or AI (17/46; 37%). AI are only prescribed in postmenopausal women. Hence, the mean age of the aromatase inhibitor group was significantly higher than the tamoxifen treated group (66.5 ± 8.1 vs. 55.5 ± 11.1; $p = 0.0009$).

3.2. Baseline levels of soluble Semaphorin 4D

At primary diagnosis, the mean baseline levels ± SD for Sema4D in the total cohort were 441.6 ± 143.4 pmol/l. All patients were then stratified according to age, menopausal status, tumor subtype, nodal status, PR/Her2 status and tumor size and mean baseline levels of Sema4D compared (Table 2). No significant differences of Sema4D plasma level were observed between patients with different tumor subtype, nodal status or tumor size. In addition, age, menopausal status or Her2/PR-positivity did not have a significant association with plasma Sema4D levels. Patients were additionally stratified according to their future endocrine treatment after surgery (AI or tamoxifen). When comparing the AI vs. tamoxifen group within clinical parameters, Sema4D levels in patients with Luminal B Her2 negative tumors in the tamoxifen group where significantly higher than in patients with Luminal A or Luminal B Her2 negative tumors in the aromatase inhibitor group ($p \leq 0.05$).

3.3. Effect of adjuvant endocrine therapy on Semaphorin 4D levels

We next analyzed the effects of adjuvant tamoxifen or AI treatment on plasma Sema4D by comparing the respective plasma levels at

Table 1
Baseline patient characteristics.

	All (n = 46)	AI (n = 17)	Tamoxifen (n = 29)	p value AI vs. Tamoxifen
Age, years (median; range)	61 (26–85)	66 (51–85)	52 (26–72)	
Age, years (mean ± SD)	60 ± 11.4	66.5 ± 8.1	55.5 ± 11.1	0.0009
<60; n (%)	22 (47.8)	3 (17.6)	19 (65.5)	
>60; n (%)	24 (52.2)	14 (82.4)	10 (34.5)	
Weight, kg (median; range)	75 (50–115)	71 (60–105)	75 (50–115)	
Weight, kg (mean ± SD)	77 ± 15.7	76 ± 15.4	77.8 ± 16.1	ns
Menopausal status; n (%)				
Premenopausal	10 (21.7)	0 (0)	10 (34.5)	
Perimenopausal	5 (10.9)	0 (0)	5 (17.2)	
Postmenopausal	31 (67.4)	17 (100)	14 (48.3)	
ER status; n (%)				
positive	46 (100)	17 (100)	29 (100)	
negative	0 (0)	0 (0)	0 (0)	
PR status; n (%)				
positive	43 (93.5)	15 (88.2)	28 (96.6)	
negative	3 (6.5)	2 (11.8)	1 (3.4)	
Her2 status; n (%)				
positive	6 (13)	1 (5.9)	5 (17.2)	
negative	40 (87)	16 (94.1)	24 (82.8)	
Tumor subtype; n (%)				
Luminal A	33 (71.7)	14 (82.4)	19 (65.5)	
Luminal B Her2 negative	7 (15.2)	2 (11.8)	5 (17.2)	
Luminal B Her2 positive	6 (13)	1 (5.9)	5 (17.2)	
Tumor size; n (%)				
pT1	36 (78.3)	12 (70.6)	24 (82.8)	
pT2	6 (13)	3 (17.6)	3 (10.3)	
pT3-4	4 (8.7)	2 (11.8)	2 (6.9)	
Nodal status; n (%)				
Node positive	13 (28.3)	6 (35.3)	7 (24.1)	
Node negative	33 (71.7)	11 (64.7)	22 (75.9)	

ER = estrogen receptor; PR = progesterone receptor.
Her2 = human epidermal growth factor receptor 2.

primary diagnosis and after one year. Assessment one year after surgery revealed a significant decrease of Sema4D levels in patients treated with tamoxifen (464.4 ± 159 pmol/l vs. 335.8 ± 89,8 pmol/l; −27.7%;$p < 0.001$), while there were no significant changes in the aromatase inhibitor treated group (402.6 ± 110.7 pmol/l vs. 413 ± 128.4 pmol/l; +2.6%; Fig. 1a and b). The results were reflected by the difference of Sema4D levels in both the tamoxifen and AI group between the two time points (one year after surgery vs. −1 day before surgery). Here, total and relative median levels of Sema4D plasma level differences were reduced in the tamoxifen group but not in the AI

Table 2
Baseline levels of Semaphorin 4D.

	All (pmol/l)	p-value all	AI (pmol/l)	Tamoxifen (pmol/l)	p-value AI vs. Tamoxifen
All	441.6 ± 143.4		402.6 ± 107.4	464.4 ± 156.3	ns
Age, years					
<60	438.1 ± 132.0		400.8 ± 111.5	443.9 ± 130.5	ns
>60	444.8 ± 132.0	ns	403.0 ± 106.5	503.3 ± 190.1	
Menopausal status					
Premenopausal	470.7 ± 155.2		–	470.7 ± 147.2	ns
Perimenopausal	421.0 ± 113.9		–	421.0 ± 101.9	
Postmenopausal	435.5 ± 148.9	ns	402.6 ± 107.4	475.4 ± 174.8	
PR status					
positive	445.6 ± 146.7		411.3 ± 106.3	463.9 ± 159.0	ns
negative	384.3 ± 123.0	ns	337.5 ± 92.6	447.8 ± 0*	
Her2 status					
positive	410.2 ± 131.5		493.3 ± 0	393.6 ± 125.0	ns
negative	446.3 ± 147.8	ns	396.9 ± 108.2	479.2 ± 158.1	
Tumor subtype					
Luminal A[1]	428.6 ± 119.6		410.9 ± 106.4	441.7 ± 123.8	0.03 (AI[1] vs. TAM[2]) 0.05 (AI[2] vs. TAM[2])
Luminal B Her2 negative[2]	529.4 ± 236.6		299.2 ± 59.3	621.5 ± 190	
Luminal B Her2 positive[3]	410.2 ± 131.5	ns	493.3 ± 0*	393.6 ± 125	
Tumor size					
pT1	437.7 ± 151.6		383.8 ± 90.9	464.7 ± 165	ns
pT2	500.0 ± 112.6		521.3 ± 90.9	478.7 ± 109.3	
pT3-4	388.5 ± 122.1	ns	337.5 ± 92,6	439.4 ± 92.8	
Nodal status					
Node positive	436.4 ± 112.9		441.2 ± 122.5	432.2 ± 94.5	ns
Node negative	443.6 ± 157.3	ns	381.5 ± 91,5	474.7 ± 170	

ER = estrogen receptor; PR = progesterone receptor.
Her2 = human epidermal growth factor receptor 2.

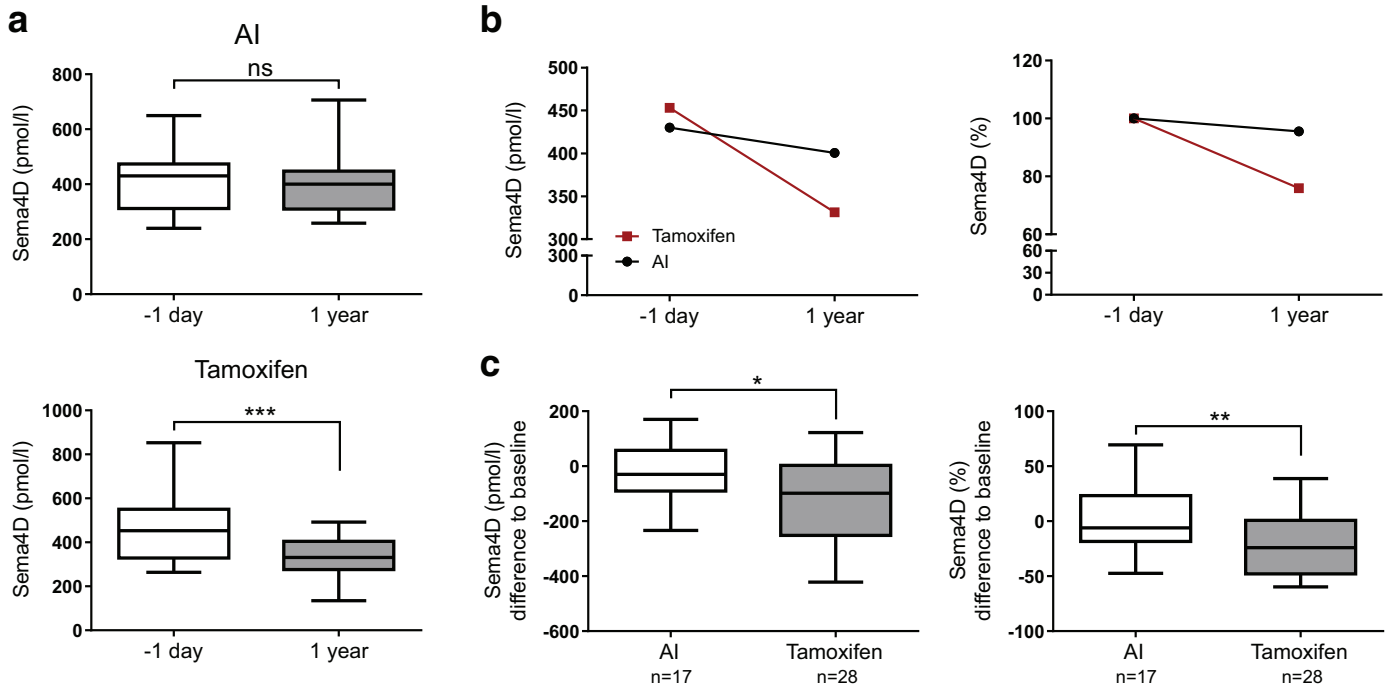

Fig. 1. Follow up of Semaphorin 4D (Sema4D) levels.
The plasma concentrations of Sema4D in the tamoxifen and aromatase inhibitor (AI) group between one day before and one year after surgery are presented as box-whisker blot (a). Of note, one patient was switched from tamoxifen to anastrazole after a short period and considered as an aromatase inhibitor patient in the analyses. In the tamoxifen group, one value for Sema4D levels one year after surgery was identified as a significant outlier and not included for the follow-up analyses. The absolute and relative follow-up values of Sema4D for the two time points are depicted as median (b). The individual and relative differences in both patient groups between one day before and one year after surgery are presented as box-whisker blot (**c**). Outliers were excluded as described in methods section. A student's *t*-test was used to perform single group comparisons. $*p<0.05$; $**p<0.01$; $***p<0.001$.

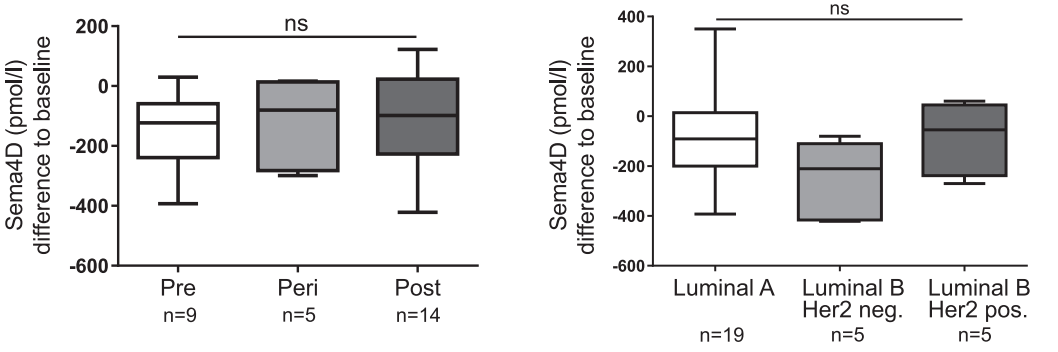

Fig. 2. Semaphorin 4D (Sema4D) level difference to baseline in the tamoxifen group.
Tamoxifen treated patients were stratified according to menopausal status and tumor type. The individual differences of Sema4D levels between one day before and one year after surgery are presented as box-whisker blot. Outliers were excluded as described in methods section. Group analyzes were performed using one-way analysis of variance (ANOVA).

group. (Fig. 1c; $p<0.01$). All patients treated with AI were postmenopausal. In the tamoxifen group, patients had varying menopausal status ranging from pre- (9/28), peri- (5/28) to postmenopausal (14/28). To exclude any effects mediated by menopausal or tumor type status, we stratified the tamoxifen group according to menopausal status or tumor type and depicted the Sema4D differences to baseline (Fig. 2). Here, no significant differences were observed between the groups.

4. Discussion

Maintaining long-term bone health in patients with breast cancer is an important issue in their clinical management. First, breast cancer cells show a high propensity to metastasize to bone where they establish osteolytic lesions in the majority of patients in advanced stages of the disease [13,14]. In addition, anti-hormonal therapies may negatively affect bone as they lead to a deprivation of bone protective estrogen and cancer treatment-induced bone loss [15]. It is widely recognized that AI and tamoxifen exert different effects on bone. AI are inhibitors of the aromatase enzyme, which is important for the conversion of estrogens from androgens in peripheral tissues including fat and breast. AI are used in postmenopausal women with hormone receptor positive breast cancer to block residual estrogen levels [16]. Hence, AI have deleterious effects on bone health [17]. By contrast, tamoxifen is a selective estrogen receptor modulator (SERM) with partial ER-agonistic and antagonistic functions, depending on the cell type [18]. In bone, tamoxifen is a partial ER-agonist and reduces bone turnover and loss in postmenopausal women [19,20]. The divergent effects of tamoxifen and AI have been described in a number of studies [21,22].

Tamoxifen is considered bone protective in postmenopausal women. As it mimics the actions of estrogen in bone, it inhibits osteoclast formation and bone resorption while osteoclastic apoptosis is increased [23–25]. Using the same cohort as in this study, we have recently revealed that adjuvant tamoxifen significantly reduced the serum levels of Dickkopf-1 (DKK-1) [12]. DKK-1 is an inhibitor of the Wnt pathway and suppresses osteoblastic differentiation thereby representing a negative player of bone formation [12]. AI therapy had no effect on serum levels

of DKK-1. In this study, we show that tamoxifen also reduced plasma levels of Sema4D in patients with hormone receptor positive breast cancer whereas no effect on Sema4D levels was seen in AI treated patients. Different effects of bone-targeted therapies on Sema4D have already described in postmenopausal women suffering from low bone mass. Circulating levels of Sema4D were increased by denosumab, but decreased following treatment with teriparatide [26]. Sema4D is produced by osteoclasts and suppresses IGF-1 signaling in osteoblasts, thereby inhibiting bone formation [2]. Hence, a reduction of circulating Sema4D by adjuvant tamoxifen therapy potentially represents an addition bone-protective property of tamoxifen in breast cancer patients.

The ELISA used in our study does not distinguish between osteoclast-derived and tumor-derived Sema4D. Studies have shown that human estrogen receptor positive breast cancer cell lines are able to express moderate protein levels of Sema4D [9]. However, given that patients in our study receiving tamoxifen in an adjuvant setting were considered tumor free, decreasing levels of Sema4D are more likely to result from effects on the bone microenvironment.

In conclusion, our study revealed a potential additional bone-protective mechanism of tamoxifen in patients with primary breast cancer by inhibition of Sema4D. Future studies are warranted to assess the underlying molecular mechanisms and the potential of Sema4D modulating therapies. Increasing the number of patients with a balanced representation of the heterogeneous phenotypes of human breast cancer may clarify whether specific patient subpopulations specifically benefit from the Sema4D-reducing actions of tamoxifen.

Compliance with ethical standards

Disclosure of potential conflicts of interest

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Conflict of Interest: The authors have received research grants or honorarium for advisory boards or lectures to the individual or the institution by Alexion (LCH), AstraZeneca (PW), Amgen (PW, LCH, TDR, TL), Novartis (PW, TL), MSD (PW, TL), Pfizer (PW, TL), PharmaMar (PW), Roche (PW, TDR, TL), TEVA (PW), Eisai (PW), Clovis (PW), Shire (LCH, TDR), Tesaro (PW), and UCB (LCH, TDR). AG, JDK, DB, SD, GF, and CLR declare no conflict of interest.

Research involving human participants and/or animals

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Authors` roles: Study Design: AG and TDR. Study conduct: AG, JDK, TL and DB. Data collection: AG, JDK, TL. Data analysis: AG, JDK, and TDR. Data interpretation: AG, JDK, PW, ST, SD, GF, DB, MR, LCH and TDR. Drafting Manuscript: AG and TDR. Revising manuscript content: AG, JDK, TL, PW, ST, SD, GF, DB, MR, LCH and TDR. Approving final version of manuscript: AG, JDK, TL, PW, ST, SD, GF, DB, MR, LCH and TDR. AG takes responsibility of the integrity of the data analysis.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2019.100237.

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