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ORIGINAL ARTICLE

Tranexamic acid can inhibit tongue squamous cell carcinoma invasion *in vitro*

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OBJECTIVES: Tranexamic acid (TA) is an inhibitor of plasminogen activation commonly used in surgery. Plasmin, the end product of plasminogen activation, degrades fibrin in the thrombus, leading to thrombolysis. However, plasmin is also associated with progression of several cancers and with cancer-associated matrix metalloproteinase-9 (MMP-9) activation. As the gelatinases MMP-2 and -9 are involved in cancer progression, several antigelatinolytic drugs have been developed as potential anticancer therapeutics. We previously developed gelatinases targeting peptide CTT1 capable of inhibiting carcinoma growth.

STUDY DESIGN: The effects of TA and CTT1 on tongue carcinoma aggressiveness were evaluated in an *in vitro* assay of human HSC-3 and SCC-25 cells.

MATERIALS AND METHODS: The cells were cultured with or without TA and CTT1 and their proMMP-9 production and activation were analysed with Western immunoblotting and gelatin zymography. Their effects on tongue carcinoma invasion were analysed in a Matrigel assay.

RESULTS: Tranexamic acid alone and in combination with CTT1 can inhibit tongue SCC invasion *in vitro*, at least partially explained by its property of reducing the plasmin-mediated activation of proMMP-9.

CONCLUSIONS: These data suggest that patients undergoing surgical therapy for large oral malignancies may cobenefit from prolonged TA therapy, because of its antithrombolytic and antitumour properties.

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Keywords: tranexamic acid; human tongue squamous cell carcinoma; invasion; migration; MMP-9

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Introduction

In case of injury, rapid and complicated cascades occur to minimize bleeding and initiate wound healing (Hall, 2006). As a result, a blood clot or thrombus is formed that consists of platelets and blood cells attached to a fibrin framework (Rang *et al*, 1999; Hall, 2006). In thrombus breakdown (thrombolysis), the events are slower and involve fewer biological components. One of the key thrombolytic steps is the activation of plasminogen to plasmin, leading to degradation of the fibrin framework in blood clots (Chapman, 1997; Rang *et al*, 1999). Optimal surgical technique, suturing and cauterization play the most important roles in the prevention of excess blood loss but pharmacological intervention in thrombus formation or prevention of its breakdown is crucial especially in dealing with the patients having bleeding disorders. Tranexamic acid (TA) is a competitive inhibitor of plasminogen activation and has been used for years in the management of peri- and postoperative bleeding (Dunn and Goa, 1999). TA is a well-tolerated drug and can be administrated systemically, either intravenously or orally, the dosage usually being 10 mg kg^{-1} (Dowd *et al*, 2002; Yaniv *et al*, 2006) but concentrations over 10 times higher have also been used (Karski *et al*, 1995). Systemic and topical application of TA is useful in the management of postoperative bleeding in maxillofacial surgery (Webster and Wilde, 2000; Frachon *et al*, 2005; Yaniv *et al*, 2006).

Plasmin is a proteolytic enzyme and its activation enhances modification and destruction of distinct intercellular and basement membrane proteins often associated with growth and spread of several malignancies (Andreasen *et al*, 1997; Hervio *et al*, 2000; Gosalia *et al*, 2006). The plasminogen activation cascade is also upregulated in various malignancies, including oral cancer (Bizik *et al*, 1990; Clayman *et al*, 1993; Tang *et al*, 2001; Curino *et al*, 2004; Murthi *et al*, 2004). Another interesting, but less focused, association of plasmin with cancer aggressiveness is its ability to activate promatrix metalloproteinase-9

(proMMP-9) (Juarez *et al*, 1993; Devy *et al*, 1997; Festuccia *et al*, 1998), although its relevance *in vivo* was also questioned (Ra and Parks, 2007). Few experimental anticancer strategies have been planned that were based on inhibition of plasminogen activation by TA (Kodama and Tanaka, 1981; Teodorczyk-Injeyan *et al*, 1983; Kikuchi *et al*, 1986), but to the best of our knowledge, no results are available from clinical trials focusing on the anticancer properties of TA. In this regard, the combination of plasminogen activation inhibitors and MMP inhibitors as anticancer therapeutics can be one potential option (Stonelake *et al*, 1997; Lacroix *et al*, 2000). However, it is uncertain whether plasminogen activation inhibitors under physiological conditions can interfere with plasmin-mediated proMMP-9 activation.

Cancer therapies and surgical techniques have been developed in recent decades, but the 5-year survival rates of oral carcinoma patients are still modest. Because of the aggressive nature of oral carcinomas, surgical treatments are often rather radical and the future development of the additional anticancer strategies is warranted. As the gelatinases MMP-2 and -9 are associated with oral carcinoma growth and spread (Juarez *et al*, 1993; Rosenthal and Matrisian, 2006), the aim of our study was to investigate the effects of the combination of TA with the selective gelatinase inhibitor CTT1 (Koivunen *et al*, 1999; Heikkilä *et al*, 2006) on the migration and invasion of squamous cell carcinoma (SCC) cells *in vitro* to determine potential supplemental therapies for oral carcinomas.

Materials and methods

Cells and cell cultures

The human tongue SCC cell lines HSC-3 (Japanese Collection of Research Bioresources Cell Bank 0623, National Institute of Health Sciences, Tokyo, Japan) and SCC-25 (American Type Culture Collection, Manassas, VA, USA) were cultured in culture flasks (Nunc, Kamstrupvej, Denmark) or 24-well Falcon plates (Becktor Dickinson, Le Pont de Claix, France) in 1:1 Ham's F12 nutrient mixture and Dulbecos modified Eagle's medium DMEM (both Lonza, Verviers, Belgium) supplemented with 2% human serum obtained from author JS (to obtain the natural components of plasminogen activation present *in vitro*), 100 U ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin (Sigma, Ayrshire, UK), 1 mM sodium pyruvate, 2 mM L-glutamine (Lonza), 0.5 nM phorbol 12-myristate 13-acetate (AG Scientific, San Diego, CA, USA), 0.4 ng ml^{-1} hydrocorticone (Diosynth, Oss, The Netherlands) and 1.25 $\mu g\ ml^{-1}$ amphotericin B (Gibco, Paisley, UK). In cell viability studies, the cells were grown in the same culture media as above but the Ham's and DMEM culture media contained no phenol red (both from Gibco). The cells were subcultured by detaching them with 1 mM cold ethylendiaminetetraacetic acid, washed with culture medium, centrifuged and resuspended in fresh culture medium.

Chemicals

Tranexamic acid was purchased in a 100 mg ml^{-1} solution (Leiras, Helsinki, Finland) and further diluted with cell culture medium or saline. The test concentration used (6 mM; 1 mg ml^{-1}) was carefully selected to reflect therapeutically attainable plasma concentrations (Karski *et al*, 1995; Dowd *et al*, 2002) that do not exert cytotoxic effects (Kikuchi *et al*, 1986; Cox *et al*, 2003). The antigelatinolytic CTTHWGFTLC peptide was custom-made to 90% purity (Neosystems, Strasbourg, France) and 1–10 mg ml^{-1} working solutions were prepared from lyophilized peptides as described (Koivunen *et al*, 1999; Heikkilä *et al*, 2006).

Recombinant enzymes

Human recombinant progelatinase B (proMMP-9) was purchased from Invitek (Berlin, Germany).

Gelatin zymography, Western immunoblotting and SDS-PAGE

The HSC-3 and SCC-25 cells were cultured with or without 6 mM TA and 100 μM CTT1 for 48 h (the TA concentration was selected to resemble potential clinical plasma concentrations) (Karski *et al*, 1995; Dowd *et al*, 2002). Production and activation of MMP-9 in the culture media were detected with gelatin zymography and Western immunoblotting as described elsewhere (Heikkilä *et al*, 2006) and the results were quantified using a Bio-Rad GS-700 densitometer and the QuantityOne program (Bio-Rad Laboratories, Hercules, CA, USA). All the experiments were performed using culture media containing 2% human serum. For detection of the effect of TA on proMMP-9 activation or activity, human recombinant proMMP-9 and 6 mM TA were incubated with β-casein with or without 1 mM 4-aminophenylmercuric acetate (APMA; Sigma) and the β-caseinolysis and MMP activation was characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Nyberg *et al*, 2003).

MTT cell viability assay

The possible effects of TA and CTT1 treatment on HSC-3 and SCC-25 viability were analysed using an MTT-assay (Sigma, St Louis, MO, USA) as described elsewhere (Koivunen *et al*, 1999). Briefly, the passaged cells were allowed to attach to the culture wells overnight, TA and CTT1 were added to the culture medium, MTT reagent was added after the incubation for 36 h and the incubation was continued for 4 h. The formazan crystals formed in the viable cells were dissolved with MTT solvent and the number of viable cells analysed using ELISA-reader (absorbance 570 nm; Labsystems, Helsinki, Finland).

In vitro *cell invasion and migration*

The effects of TA and CTT1 on SCC invasion and migration potential were studied, using 8.0-μm pore size Matrigel™ 24-well plate invasion chambers and control inserts of equal size (BD Biosciences, Bedford, MA, USA) (Heikkilä *et al*, 2006). Briefly, CTT1 was diluted to 1 mM in H_2O and the desired concentration with TA

was prepared in culture medium, cells (2×10^5 per well) and inhibitors were preincubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere, culture medium containing 10% human serum was then added to the lower compartment of the chamber, creating a chemoattractor serum gradient, and incubation continued for 48 h. The cells were methanol-fixed, washed with tap water, stained with 1% toluidine blue and the non-migrated fraction removed with a cotton swab. Quantitative analysis was performed with the Bio-Rad GS-700 imaging densitometer.

Analysis of the data

All the proteolytic and *in vitro* experiments were repeated three times and the data are expressed as mean ± s.d. The effects on cell migration and invasion are represented as percentages and the statistical significances were analysed with Student's *t*-test. Results with $P < 0.05$ were regarded as significant.

Results

TA and CTT1 treatments reduced HSC-3 cell motility in vitro

Tranexamic acid alone at 6 mM and especially in combination with 100 μM CTT1 effectively reduced HSC-3 cell transmigration to 64 ± 7% ($P = 0.0016$) and 55 ± 8% ($P = 0.0007$) relative to the controls (Figure 1a). As expected, CTT1 alone at 100 μM reduced HSC-3 cell migration to 71 ± 12% ($P = 0.01$). TA, CTT1 or their combination did not affect SCC-25 cell migration ($P = 0.36$, 0.27 and 0.46 respectively) (Figure 1a).

TA and CTT1 treatments inhibited HSC-3 and SCC-25 cell invasion in vitro

Tranexamic acid at 6 mM significantly inhibited HSC-3 ($P = 0.036$) and SCC-25 ($P = 0.0024$) cell invasion in matrigel (by 36 ± 13% and 52 ± 5% respectively), whereas the 100 μM CTT1 treatment alone, as expected, reduced the HSC-3 and SCC-25 cell invasion by 35 ± 10% and 52 ± 1% ($P = 0.035$ and 0.0018 respectively) (Figure 1b). The combination of 6 mM TA with 100 μM CTT1 potentiated the inhibitory effect: the HSC-3 and SCC-25 cell invasion potential was reduced by 52 ± 5% and 59 ± 3% ($P = 0.0079$ and 0.001 respectively) (Figure 1b).

Cell viability was unaffected by TA and CTT1 treatments

As there are studies showing that TA has cytotoxic effects on some cells (Kikuchi *et al*, 1986; Cox *et al*, 2003), although more than five times higher concentrations, we analysed the TA and CTT1 treatment effects on HSC-3 and SCC-25 cell viability, using the MTT assay. TA at 6 mM alone or combined with 100 μM CTT1 did not affect HSC-3 or SCC-25 cells viability during a 36-h incubation (Figure 1c).

TA combined with CTT1 inhibited proMMP-9 activation in cell culture

The HSC-3 and SCC-25 tongue carcinoma cells were allowed to attach to 24-well plates overnight, after

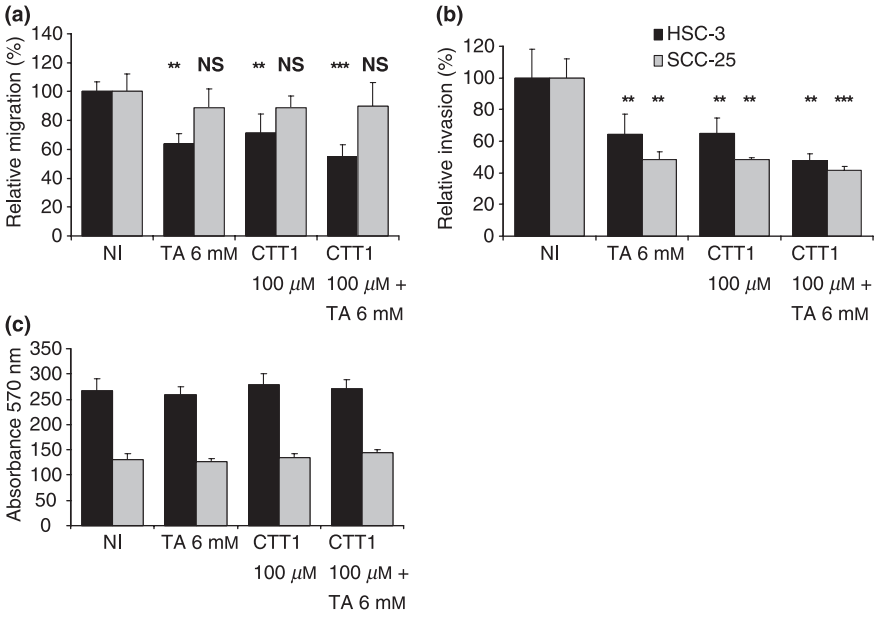

Figure 1 Effect of tranexamic acid (TA) and CTT1 on HSC-3 and SCC-25 cell invasion *in vitro*. TA at 6 mM and antigelatinolytic CTT1 peptide at 100 μM effectively inhibited HSC-3 cell transmigration in 8.0-μm pore size culture inserts, compared with controls in which no inhibitor (NI) was used (**a**). When the TA and CTT1 therapies were combined, the HSC-3 transmigration was reduced almost to half ($P = 0.0007$). SCC-25 cell migration was unaffected by the TA and CTT1 therapies. TA and CTT1 in the same concentrations as above effectively inhibited both HSC-3 and SCC-25 cell invasion in an 8.0-μM pore sized Matrigel assay (**b**). Combining TA with CTT1 was the most effective option: the HSC-3 and SCC-25 cell invasion potential was reduced to under half ($P = 0.0079$ and 0.001 respectively). The effect of these therapies on cell viability was tested by incubating HSC-3 and SCC-25 cells with or without 6 mM TA and 100 μM CTT1 or their combinations for 36 h. These therapies did not affect HSC-3 or SCC-25 cells viability (**c**). The experimental data are expressed as mean values ± s.d. of three independent experiments

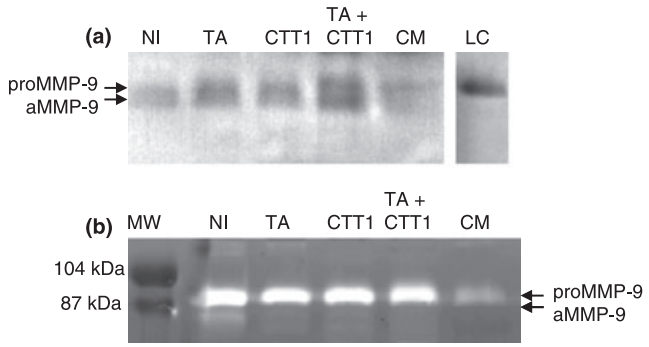

Figure 2 Effect of TA and CTT1 on MMP-9 conversion of SCC-25 culture media. As demonstrated in Western immunoblotting, both CTT1 at 100 μM and TA at 6 mM, as expected, reduced the conversion of the 92-kDa proMMP-9 to the 82-kDa active MMP-9 in SCC-25 culture medium, compared with control medium in which no inhibitor (NI) was used (**a**). However, the effect was not statistically significant. Combination of TA with CTT1 therapies clearly inhibited the proMMP-9 activation ($P = 0.035$). NI again represents the serum-containing medium from the control cell culture, CM the 2% human serum-containing medium without cells which mainly consists of proMMP-9 and LC the loading control of human recombinant proMMP-9. The inhibitory effect was more clearly seen in gelatin zymography (**b**). The data consist of mean values ± s.d. of three independent experiments

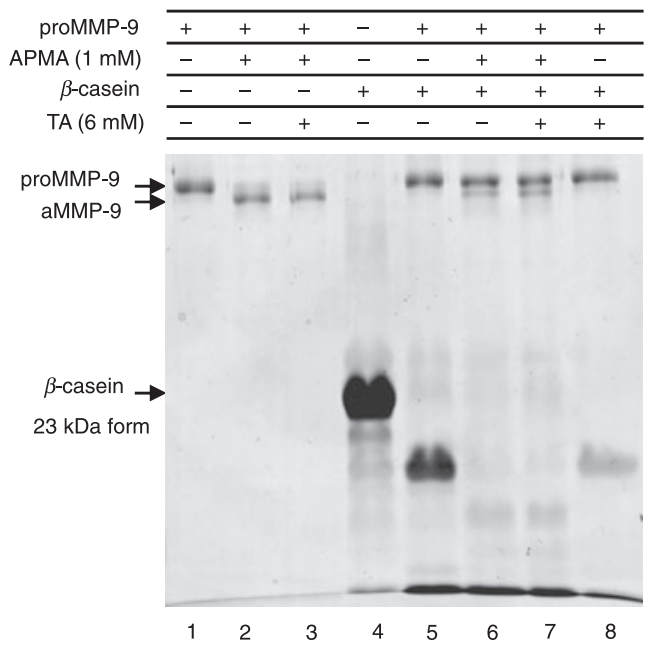

Figure 3 Effect of TA on recombinant proMMP-9 activation. As proMMP-9 activation was reduced by 6 mM TA treatment in oral tongue SCC cultures, the effects of TA on MMP-9 activation and activity were further tested. Recombinant human proMMP-9, the artificial activator 4-aminophenylmercuric acetate (APMA), and β-casein were assayed with or without TA. Lane 1 represents human recombinant proMMP-9 alone, in which only a small fraction can be seen as converted to the active form. APMA activates proMMP-9 (lane 2) and TA at 6 mM did not inhibit this process (lane 3). Lane 4 represents the MMP-9 substrate β-casein alone. Supplementation of proMMP-9 with β-casein already results in moderate β-caseinolysis (lane 5), whereas APMA-activated MMP-9 results in total fragmentation of β-casein (lane 6). TA at 6 mM did not affect the β-caseinolytic activity of active MMP-9 (lane 7), whereas it slightly enhanced the β-casein fragmentation by proMMP-9 (lane 8), compared with proMMP-9 alone (lane 5)

which the culture medium was replaced with either fresh culture medium alone or supplemented with 6 mM TA, 100 μM CTT1 or their combinations and the cells allowed to grow again for 48 h. MMP-9 production and activation from the collected culture medium were detected, using Western immunoblotting (Figure 2a) and gelatin zymography (Figure 2b). In the Western immunoblot, the combination of TA and CTT1 reduced the proMMP-9 conversion in the SCC-25 cell culture medium to 71 ± 8% from the controls ($P = 0.035$). CTT1 inhibits proMMP-9 activation (Heikkilä *et al*, 2006), in addition TA and CTT1 in separate incubations could reduce SCC-25 proMMP-9 conversion to 83 ± 12% and 94 ± 7%, respectively, but the quantitative results were not statistically significant ($P = 0.18$ and 0.54). In the HSC-3 cell medium, only a small fraction of the 92-kDa proform of MMP-9 was converted to the active 82-kDa form, which may explain why the effects of TA were difficult to determine using densitometry (not shown).

APMA activation and activity of pure human recombinant MMP were unaffected by TA

To determine whether TA can directly affect proMMP activation, recombinant human proMMP-9 was incubated with or without 6 mM TA and 1 mM APMA. In addition, the possible effects of TA on the proteolytic activity of MMP-9 were assayed, using β-casein as an MMP-9 substrate. TA at 6 mM did not affect APMA-induced proMMP-9 activation or MMP-9 proteolytic activity (Figure 3).

Discussion

The plasminogen activation cascade is associated with the progression, aggressiveness or poorer prognosis of several cancers (Andreasen *et al*, 1997; Festuccia *et al*, 1998; Tang *et al*, 2001; Choong and Nadesapillai, 2003; Murthi *et al*, 2004). As the main physiological role of plasminogen activation is considered to be related to thrombolysis, and many other prominent proteolytic enzymes also affect cancer progression, little interest has been shown in TA as an anticancer therapeutic. Plasmin, the end product of the plasminogen activation cascade, is also capable of activating proMMP-9 (Juarez *et al*, 1993; Devy *et al*, 1997; Festuccia *et al*, 1998); however, some data also suggest that this activation may not have physiological relevance *in vivo* (Ra and Parks, 2007). The MMPs, particularly the gelatinases MMP-2 and -9, are associated with the aggressiveness of oral SCCs (Juarez *et al*, 1993; Hong *et al*, 2000; Ruokolainen *et al*, 2005; Heikkilä *et al*, 2006; Rosenthal and Matrisian, 2006). Our previous studies with selective gelatinase inhibitors demonstrated that gelatinase inhibition with small cyclic peptides may inhibit cancer progression *in vitro* and *in vivo* (Koivunen *et al*, 1999; Heikkilä *et al*, 2006).

Recent studies with selective plasmin inhibitors also yielded promising results as possible anticancer therapeutics (Devy *et al*, 2007). We demonstrated here that TA alone, and especially when combined with peptides

that target MMP-9 (Koivunen *et al*, 1999; Heikkilä *et al*, 2006), can effectively inhibit HSC-3 and SCC-25 tongue carcinoma cell invasion *in vitro*. In this regard, 6 mM or lower TA concentration in combination with CTT1 might downregulate experimental tumour growth in mice and further confirms the culture result. TA with or without CTT1 could also reduce the proMMP-9 conversion to active MMP-9 in the SCC-25 cell culture, but did not affect the APMA-induced activation or activity of pure recombinant human MMP-9. When TA and CTT1 were combined, the proMMP-9 conversion in the SCC-25 culture was significantly reduced, indicating the presence of indirect synergistic effects. These data suggest that the anticancer potential of TA, at least partially, may be explained by its ability to reduce plasmin-mediated activation of proMMP-9.

The proMMP-9 conversion to active MMP-9 was not totally inhibited in the SCC-25 culture and was almost unaffected in the HSC-3 cell culture, which is an interesting finding because these cell lines express relatively equivalent amounts of proMMP-9. HSC-3 and SCC-25 cells can produce a variety of cell surface receptors and proteinases involved in their basement membrane interactions and transmigration (Ramos *et al*, 1997; Väänänen *et al*, 2001; Moilanen *et al*, 2002) and certain malignant cell lines are also capable of producing endogenous components of the plasminogen activation cascade (Hasina *et al*, 2003). Therefore, we cannot exclude the possibility that TA's anti-invasive and antimigratory potential may also be explained by its other known and as yet unknown properties.

Tranexamic acid is a widely used and well-tolerated drug (Dunn and Goa, 1999), and our present data suggest that it may inhibit tongue carcinoma *in vitro*, especially when combined with the selective antigelatinolytic peptide CTT1. TA exhibits a relatively short half-life in plasma and its anticancer potential is still rather modest, which suggests that its usage alone as an anticancer therapeutic most probably would not be successful. Interestingly, TA is tolerated in quite high dosages and the plasma concentrations gained correspond to the 6 mM used in our experiments, suggesting potential clinical relevance (Karski *et al*, 1995; Dowd *et al*, 2002). Further clinical trials using TA combined with conventional anticancer drugs are needed to evaluate its potential anticancer effects. In this regard, patients undergoing massive surgical resections of oral malignancies could possibly cobenefit from prolonged high-dose intravenous TA therapy, because of its antithrombolytic and anticancer effects. As TA exerts antimigratory potential, it could also be used to reduce extravasation of shedding carcinoma cells during surgical excision of the cancer. For the reasons stated above combination therapy using antigelatinolytic peptides, TA and conventional cytotoxic drugs may be beneficial for treatment of oral carcinomas in addition to surgery.

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Conflicts of interest

None.

Author contributions

DDS, BM J. Suojanen was responsible for study design, experimental setup and data analysis. Prof. T. Sorsa and T. Salo are responsible for study group leadership, data analysis and reporting.

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