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# Cholinergic Signaling Mechanisms and Early Implant Healing Phases in Healthy Versus Generalized Aggressive Periodontitis Patients: A prospective, case-control study

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## ABSTRACT

### Aims

Periodontal diseases negatively affect implant osseointegration. Perturbations in non-neuronal cholinergic signaling mechanisms are associated with periodontitis; however, their role in generalized aggressive periodontitis (GAgP) is unknown. The aim of this prospective case-control study was to determine the relationship between non-neuronal cholinergic signaling mechanisms, Secreted Ly-6/uPAR-related protein-1 (SLURP-1), Interleukin-17 (IL-17) family cytokines and healing of dental implants in health and GAgP**.**

### Materials and Methods

Thirteen GAgP patients and seven periodontally healthy individuals (PH) were recruited. Peri-implant crevicular fluid (PICF) was obtained at baseline and 1-month post-placement. Acetylcholine (ACh) levels and cholinesterase activity were determined biochemically. SLURP-1, IL-17A and IL-17E levels were determined by ELISA. Marginal bone loss (MBL) at 1- and 6-month(s) post-placement was determined radiographically.

### Results

The concentration of ACh, cholinesterase activity and IL-17A levels were elevated in PICF of patients with GAgP compared to PH individuals at baseline and 1-month post-placement. The concentration of ACh and cholinesterase activity levels in PICF correlated with levels of IL-17A and MBL around implants 1-month post-placement in patients with GAgP.

**Conclusions**

Non-neuronal cholinergic mechanisms may play a role in the aetiopathogenesis of GAgP and may directly or indirectly, through modulation of IL-17A, influence early implant osseointegration and potential long-term implant survival.

## CLINICAL RELEVANCE

### Scientific Rationale

Perturbations in cholinergic signaling mechanisms correlate with alveolar bone loss in periodontitis. The role of cholinergic signaling in healing of implants placed in GAgP patients is unknown.

### Principal Findings

Acetylcholine levels, IL-17A levels and cholinesterase activity were elevated in PICF of patients with generalized aggressive periodontitis. Acetylcholine levels and cholinesterase activity in PICF of patients with GAgP correlated with IL-17A levels and marginal bone loss around implants 1-month post-placement.

### Practical Implications

The data demonstrates the need for further study into the role of cholinergic mechanisms and IL-17A in modulating early osseointegration events in patients with GAgP.

## INTRODUCTION

Dental implants are a widely used treatment option for both fully and partially edentulous individuals. Periodontally compromised patients also benefit from dental implants for replacement of their missing teeth. Implant survival ratio is no longer considered as a challenge. Indeed, contemporary dental implants have on average a 95 - 100% rate of 5 - 10-year survival depending on the implant design (Buser et al. 2012, Karl and Albrektsson. 2017, Dong Mei et al. 2018). However, it is well documented that over this time, approximately 38% of dental implants exhibit complications (Pjetursson et al., 2007); and susceptibility to periodontal diseases is one of the major factors that influence long-term implant survival (Roccuzzo et al., 2014, Roccuzzo et al., 2010, Gatti et al., 2008).

There are a limited number of studies that have specifically evaluated survival rates of implants in patients having the clinical diagnosis of GAgP (Swierkot et al., 2012, Mengel et al., 2007). Anitua et al. (2008) reported that 69% of patients with implant failure presented with a history of chronic periodontitis or GAgP. Furthermore, patients with GAgP showed an implant survival rate of just 83.3% and significantly greater marginal bone loss than their periodontally healthy counter parts (Mengel et al., 2007). Patients with GAgP were 5 times more likely to experience implant failure and 14 times more likely to experience peri-implantitis than periodontally healthy individuals (Swierkot et al., 2012). Moreover, initial clinical periodontal diagnosis (e.g. GAgP vs. chronic periodontitis) has been suggested to effect implant failure rate (Monje et al., 2014). One reason may be the higher marginal bone loss observed in patients with GAgP during the first year after implant placement (Mengel et al., 2007). However, the exact mechanisms of higher marginal bone loss around dental implants in patients with GAgP have yet to be clarified.

Cholinergic mechanisms exist in the periodontium and acetylcholine (ACh) has been found to play important roles in both periodontal health and disease (Nguyen et al., 2000, Arredondo et al., 2003, Zoheir et al., 2012). ACh is synthesised by cells of the periodontium expressing choline acetyltransferase (ChAT) from acetyl COA and choline (Wessler and Kirkpatrick, 2008). However, an important determinant of levels of ACh in biofluids are the levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BCHE) activity (Pope and Brimijoin, 2018).

In health, there is a delicate balance between ACh synthesis and degradation (Nizri et al., 2007). Perturbations in this balance play roles in the aetiopathogenesis of inflammatory diseases (Fujii et al., 2017, Hoover, 2017). ACh acts through 2 major receptor subtypes; nicotinic (nAChR) and muscarinic receptors (mAChR) (Wessler and Kirkpatrick, 2008). ACh has anti-inflammatory properties through activation of ACh receptors; in particular the alpha 7 nicotinic receptor (α7nAChR) (Rosas-Ballina and Tracey, 2009). However, ACh, via other cholinergic receptor subtypes, can also have proinflammatory properties (Kistemaker et al., 2012). Therefore, cholinergic mechanisms may ‘fine tune’ the immune response and promote effective clearance of pathogenic threats whilst limiting bystander tissue damage (Rajendran et al., 2015).

Secreted Ly-6/uPAR-related protein 1 (SLURP-1) is a member of the Ly6/uPAR family of proteins and shares some structural homology with nAChR agonists (Vasilyeva et al., 2017). SLURP-1 can act as allosteric antagonist of the α7nAChR but has also been demonstrated to activate metabotropic-signalling pathways

(Lyukmanova et al., 2016). SLURP-1 is secreted at mucosal sites and has also been shown to have both immunomodulatory (Moriwaki et al., 2007) and antimicrobial properties (Moriwaki et al., 2015). In addition, SLURP-1 has been found to inhibit proliferation of oral keratinocytes (Lyukmanova et al., 2016). However, the exact roles of SLURP-1 in oral infections have still to be delineated.

Levels of IL-17 family cytokines, particularly elevated levels of IL-17A and perturbations in the IL-17A:IL-17E ratio, in gingival crevicular fluid (GCF) have been shown to be associated with bone loss in periodontal diseases (Awang et al 2014). Recently, clinical evidence for an association between biofluid levels of ACh, esterase activity and periodontal diseases have also been established (Apatzidou et al., 2018). Furthermore, there is evidence to suggest that cholinergic mechanisms can modulate bone metabolism and turnover (En-Nosse et al., 2009, Kauschke et al., 2015, Haupt et al., 2015, Ternes et al., 2015). However, no studies have investigated the role of cholinergic mechanisms, SLURP-1 and IL-17 family cytokines in implant placement outcomes. The aim of this research therefore was to determine the relationship between cholinergic signaling mechanisms, inflammation and the early healing phases of dental implants in health and GAgP**.**

## MATERIALS AND METHODS

### Study population

Twenty patients (13 GAgP patients and 7 periodontally healthy individuals) were recruited for this prospective, case-control study between October 2016 - May 2018 at the Department of Periodontology, School of Dentistry, Ege University. The study was conducted in full accordance with ethical principles, including the World Medical

Association's Declaration of Helsinki, as revised in 2000. The study protocol was approved by the Ethics Committee of Ege University, İzmir, Turkey (Protocol number; 16-10/2). Written informed consent was received from each patient before enrolment in the study. Detailed medical and dental histories were obtained from all participants and clinical and radiographic examinations were performed. Eligible patients had a clinical diagnosis of GAgP (Armitage, 1999). According to the new classification system, there is no longer differentiation between generalized chronic or aggressive periodontitis. Based on this new classification the patients recruited for the present study are in stage 3 (Papapanou et al., 2018). All patients were in the maintenance phase following initial treatment for at least one year with full-mouth plaque and bleeding scores <20%, sufficient bone volume for standard sized implants and no extractions in the edentulous sites in the last year. Individuals were excluded if they had history of systemic diseases, were pregnant, had physical and/or psychiatric disorders which hinder optimum plaque control and used antibiotics and/or anti-inflammatory drugs within the last six months. Periodontally healthy individuals (Offenbacher et al., 2008) in need of implant placement made up the control group. Smoking status was assigned following the criteria described by Schwartz-Arad *et al*, (2002).

**Surgical procedure**

All implants (3.8 mm in diameter & 7 or 9 mm in length, Isy, Camlog, Basel, Switzerland) were placed in the left or right sides of the maxilla/mandible under local anesthesia with a minimal flap reflection to minimize trauma to the gingival papillae. One stage surgery was performed, and gingiva formers were placed on a pre-mounted implant base. The flaps were closed with single interrupted sutures using a

5-0 propylene suturing material. After a healing period of 12 weeks, the prosthetic process was initiated. No grafting of soft or hard tissues was performed and no medication was prescribed for any patient. All implants were placed by the same periodontist (PM). The sutures were removed 10 days post-operatively. At each recall session, the patients were re-motivated and re-instructed in effective oral hygiene to maintain whole mouth plaque and bleeding scores < 20%; as assessed by visual examination.

**Prosthetic procedures**

The healing caps were removed after the osseointegration period. Conventional impression taking was performed with a polyether impression material (Impregum PentaDuoSoft H, 3M, Seefeld, Germany). Titanium bases (Ti-base for Isy, Camlog) compatible with the implant diameter were placed on the cast models and optical impressions were made (in-Eos X5, Sirona). The abutment crowns were designed and milled (Cerec MCXL, Sirona) out of lithium-disilicate glass-ceramic blocks (e.max CAD, IvoclarVivadent, Schaan, Liechtenstein). After crystallization, the crowns were adhesively luted to titanium bases by a self-adhesive resin cement (Multilink Hybrid Abutment Cement, IvoclarVivadent). After removing excess cement, the abutments were sterilized in an autoclave for 15 min at 121°C. A 30-Ncm torque was applied on the implant and the screw hole was restored with a composite resin to further isolate the adhesive area from the oral environment. All prosthetic procedures were performed by the same prosthodontist (EÇ). The patients were informed and educated on maintaining an optimal oral hygiene regimen.

**Radiographic analysis**

Marginal bone level (MBL) was evaluated on the standardized periapical radiographs taken using a film holder (Super-Bite film-holding system [Kerr Corporation, Orange, CA, USA]) at 10-days, 1-month and 6-months post-surgery by using the long cone paralleling technique. Care was taken to parallel the alignment of the X-ray film in the film holder to the long axis of the implants. Images were taken with an intra oral radiation unit using an acylindrical tube head, 2.5mm aluminium filtration and a focal spot distance of 200 mm. The exposure settings were 70 kV and 1.12 mAs. Images were transferred to the computer by a photostimulatable phosphor plate scanner (Digora Optime, Soredex, Milwaukee Wisconsin). Implant lengths were used as the reference for measurements on each image. MBL was calculated at the mesial and distal implant surfaces by measuring the distance between the most coronal point of the implant and the most coronal radiographic bone-implant contact with the image analysis software program (ImageJ, for Windows, NIH, Bethesda, MD). Bone loss at 1-month and 6-months was calculated by subtracting the MBL at these time-points from the MBL at baseline (10-days after implant placement). Mean values from triplicate measurements were calculated. All measurements were performed by a single calibrated examiner (PM).

**Biofluid sampling**

Peri-implant crevicular fluid (PICF) samples were obtained at baseline and at 1-month after implant placement. PICF was collected using paperstrips (Periopaper; ProFlow, Inc., Amityville, NY) by inserting the strip 1 mm into the crevice and leaving in place for 30 sec. Care was taken to avoid mechanical injury; samples with visual blood contamination were discarded. The PICF volume absorbed on to each paper

strip was determined by a specific electronic impedance device (Periotron 8000, ProFlow, Inc., Amityville, NY, USA) and the strip was placed in one sterile polypropylene tube. Samples were immediately frozen and stored at -40°C until the laboratory analysis. Readings from the Periotron 8000 were converted to actual volumes (µl) by reference to the standard curve.

**Quantification of Acetylcholine levels and cholinesterase activity in PICF samples**

ACh levels were quantified using the Acetylcholine Assay Kit in fluorometric mode as per manufacturers instructions (Abcam, Cambridge, UK). Assay plates were analyzed on a microplate reader (BMG-Labtech, Ortenberg, Germany) in fluorometric assay mode at Ex/Em = 535/587 nm.

AChE and BChE activity levels was determined using a modified version of the biochemical assay developed by Naik *et al* (Naik et al., 2013) as previously described (Apatzidou et al., 2018).

**Quantification of IL17A, IL17E and SLURP-1 levels in PICF samples**

Levels of IL-17A and IL-17E were determined using commercially available ELISA kits (Peprotech, UK); as previously described (Apatzidou et al., 2018). Levels of SLURP-1 were determined using an in-house ELISA. In brief, microtitre plates were coated with 3 µg/ml of rabbit polyclonal anti-SLURP-1 capture antibody (ABNOVA, Oxford, UK). The plates were then blocked with 1% BSA. A standard curve was developed using a LYNX1 (SLURP-1) overexpressed lysate (Origene, Rockville, USA) with an assay standard range of 3.13 - 400 pg/mL. One hundred µl of prepared

standards or sample were assayed in triplicate. Detection was carried using 2 µg/ml of mouse monoclonal anti-SLURP-1 detection antibody (ABNOVA, Oxford, UK) and a 1/5000 dilution of biotin labelled anti-mouse IgG (SIGMA, Poole, UK). Colour development was performed using a 1/2000 dilution of Streptavidin HRP (Peprotech, London, UK) and TMB substrate (Insight Biotechnology, Wembley, UK). The reaction was stopped with 1 M HCl and the absorbance was measured at 450 nm with wavelength correction set at 650 nm using a microplate reader (FLUOstar Omega; BMG Labtech, Germany). The limits of detection for each assay were determined as two mean standard deviations higher than the mean baseline from six replicate standard curves: IL-17A = 1.9 pg/ml; IL-17E = 3.8 pg/ml and SLURP-1 = 3.1 pg/ml.

**Statistical analysis**

Alpha was set to 5% and statistical power analysis was determined for independent sample parametric tests. To achieve 80% statistical power with an effect size of 1.5, average group sizes of n=8 would be required. For non-parametric tests average group sizes of n>9 would be necessary. However, with the same effect sizes, group sizes of n>7 would exceed 80% statistical power in dependent sample, non-parametric statistical analyses.

Cross-sectional analysis was performed on the participants comparing the measurements from implants of GAgP patients with implants from healthy individuals using the Mann-Whitney-U-test. Differences between measurements of biomarkers at baseline and 1-month were analyzed using the Wilcoxon rank test. The non-parametric Kendal Tau correlation test was used to investigate relationships between

the measured parameters. Furthermore, the relationship between MBL and biomarker levels or activity was investigated using a linear regression model.

## RESULTS

### Study population

Table 1 shows the demographic parameters and clinical outcomes for the study population. The median ages of the periodontitis patients and the healthy controls were similar (35 and 34 years, respectively). There was no significant difference in the number of smokers or in the gender distribution between the groups. The distribution of implants in the maxilla and mandible was equal. Marginal bone loss was significantly greater around implants in the GAgP group at 1-month and 6-months ($p= 0.026$ and $p= 0.019$, respectively) compared to the PH group. Marginal bone loss was increased in the GAgP patients at 6-months compared to 1-month ($p= 0.021$). However, there was no significant difference in marginal bone loss between the measurements at 1-month and 6-months in the PH group ($p>0.05$).

### Concentrations of Acetylcholine and esterase activity in PICF

ACh concentrations in PICF samples were significantly greater in the patients with GAgP than in the PH patients at baseline and 1-month (both $p< 0.001$). Concentrations of ACh were significantly higher at 1-month than at baseline in PH patients ($p= 0.013$) (Figure 1A). AChE activity was significantly greater in patients with GAgP than in PH patients at baseline and 1-month (both $p< 0.001$). AChE activity was significantly greater at 1-month than at baseline in both PH and GAgP groups ($p=0.018$ and $p<0.001$, respectively) (Figure 1B). BChE activity was

significantly greater in GAgP group than PH group at baseline and 1-month (both p< 0.001). BChE activity was significantly lower at 1-month than at baseline in PH group (p= 0.012) (Figure 1C).

**Secreted Ly-6/uPAR-related protein-1, IL-17A and IL-17E levels in PICF**

At baseline SLURP-1 levels were significantly greater in patients with GAgP than in PH patients (p= 0.022). Levels of SLURP-1 were greater at 1-month compared to baseline in both PH and GAgP groups (p=0.001 and p<0.006, respectively) (Figure 2A). At baseline and 1-month IL-17A levels were significantly greater in patients with GAgP than PH patients (both p<0.001). Furthermore, IL-17A concentrations were greater in patients with GAgP at 1-month compared to baseline (p<0.01) (Figure 2B). At baseline, IL-17E levels were significantly lower in patients with GAgP than PH patients (p=0.026). At 1-month IL-17E levels were higher than baseline in both PH patients (p=0.0425) and in patients with GAgP (p<0.001) (Figure 2C). The ratio of IL-17A:IL-17E was higher in patients with GAgP than PH patients at both baseline and 1-month (both p< 0.001) (Figure 2D).

**Correlations between biological parameters**

At baseline (Table 1A) ACh concentrations correlated with AChE activity, IL-17A levels and the IL-17A:IL-17E ratio (Tau= 0.389, p<0.001; Tau =0.363, p<0.001; Tau= 0.395, p<0.001, respectively). AChE activity correlated with IL-17A levels, the IL-17A:IL-17E ratio and SLURP-1 levels (Tau= 0.549, p<0.001; Tau= 0.435, p<0.001; Tau= 0.390, p= 0.001, respectively). BChE activity correlated negatively with IL-17E levels (Tau= -0.275, p=0.010). IL-17A levels correlated with SLURP-1 levels (Tau=

0.441, p<0.001). IL-17E levels correlated with SLURP-1 levels (Tau =0.364, p=0.001).

At 1-month (Table 1B) ACh concentrations correlated with AChE activity, IL-17A levels, IL-17E levels, SLURP-1 levels and the IL-17A:IL-17E ratio (Tau= 0.674, p<0.001; Tau= 0.713, p<0.001; Tau= 0.507, p<0.001; Tau=0.584, p<0.001; Tau=0.535, p<0.001, respectively). AChE activity correlated with BChE activity, IL-17A levels, IL-17E levels, SLURP-1 levels and the IL-17A:IL-17E ratio (Tau= 0.369, p=0.001; Tau= 0.729, p<0.001; Tau= 0.568, p<0.001; Tau=0.388, p=0.001; Tau= 0.511, p<0.001, respectively). BChE activity correlated with IL-17A levels (Tau= -0.280, p=0.011). IL-17A levels correlated with IL-17E levels, the IL-17A:IL-17E ratio and SLURP-1 levels (Tau= 0.448, p<0.001; Tau= 0.757, p<0.001 and Tau= 0.472, p<0.001, respectively). IL-17E levels and the IL-17A:IL-17E ratio correlated with SLURP-1 levels (Tau= 0.344, p=0.001 and Tau= 0.560, p<0.001, respectively).

**Correlations between biological parameters and clinical outcome**

There were statistically significant correlations between baseline ACh concentrations (Tau=0.573, p<0.001), AChE activity (Tau= 0.249, p=0.037), IL-17A levels (Tau= 0.398, p=0.001) and the IL-17A:E ratio (Tau= 0.445, p<0.001) and MBL recorded at 1-month. There were no statistically significant correlations between any of the biological parameters in PICF at 1-month with MBL at 1-month or 6-months. In a linear regression model using MBL as the determinant the only statistically significant correlations were between MBL at 1 month and baseline measurements of ACh and SLURP-1 (R=0.475, p<0.001; R=0.254, p= 0.046, respectively).

## DISCUSSION

Patients with periodontitis, or a history of periodontitis, exhibit more biological complications and a higher rate of implant loss than patients with a healthy periodontium (Roccuzzo et al., 2014, Roccuzzo et al., 2010, Gatti et al., 2008). Periodontitis can be classified as chronic or aggressive. The latter presents clinically with a rapid form of tissue destruction and is much less prevalent in the population (<1%). Although the relationship between chronic periodontitis and implant success rates has been extensively studied, to date, few studies have specifically evaluated survival rates and early osseointegration events around implants in patients with the clinical diagnosis of GAgP.

In this study, we report for the first time that levels of ACh, SLURP-1 and both AChE and BChE activity were elevated in PICF samples of patients with GAgP compared to those with a healthy periodontium at both baseline (10 days after implant placement) and 1-month. A linear regression model where MBL was used as the determinant substantiated the finding as baseline ACh and SLURP-1 levels correlated with MBL at 1-month. Furthermore, non-parametric correlation analysis indicated that baseline ACh concentration and AChE activity levels correlated with MBL recorded at 1-month. This is in agreement with a previous study which demonstrated that ACh levels and esterase activity in saliva and gingival crevicular fluid correlated with alveolar bone loss in chronic periodontitis (Apatzidou et al., 2018).

The relationship between esterase activity and bone metabolism has been investigated previously. Knock-out of *AChE* and *BChE* in mice had no major significant effects on bone parameters (Kauschke et al., 2015, Haupt et al., 2015). However, in *AChE* knock-out mice the number of osteoclasts per perimeter was significantly reduced in lumbar vertebrae (Kauschke et al., 2015) and in *BChE* knock-out mice a significant increase in relative osteoclast number was observed (Haupt et al., 2015). The role of ACh in bone metabolism remains unclear. Non-neuronal cholinergic systems are present in both osteoclasts and osteoblasts and suggested to play a role in bone turnover (En-Nosse et al., 2009, Ternes et al., 2015). Indeed, osteoblasts express both nicotinic (nAChRs) and muscarinic ACh receptors (mAChRs) and ACh has been shown to regulate their proliferation and differentiation (Sato et al., 2010). In contrast, ACh did not have any direct activity on osteoclasts *in vitro* (Ternes et al., 2015). However, *in vivo*, the alpha 7 nicotinic receptor (α7nAChR) on macrophages has been demonstrated to regulate bone turnover by inhibiting TNF-a expression and subsequently down regulating osteoblastogenesis and promoting osteoclast activity (Mito et al., 2017). In terms of SLURP-1, there is no direct evidence for a role in hard tissue homeostasis however, as an activator of the α7nAChR it has been shown to indirectly regulate the ability of deciduous dental pulp stem cells DDPSCs to influence osteoclastogenesis and enhance resorption (Wang et al., 2017). Therefore, the evidence suggests that cholinergic mechanisms can have both direct and indirect effects on bone metabolism; the latter through modulation of the host immune response.

In this study, levels of IL-17A and IL-17E, as well as the IL-17A:IL-17E ratio are all elevated in PICF of patients with GAgP at baseline. Furthermore, levels of IL-17A and the IL-17A:IL-17E ratio are elevated in PICF of patients with GAgP compared to those with a healthy periodontium at 1-month. Indeed, there were statistically significant correlations between baseline IL-17A levels and the IL-17A:E ratio with MBL recorded at 1-month. In addition, there are moderate to strong statistically significant correlations between ACh concentration and AChE activity with IL-17A levels in PICF at baseline and 1 month.

The role of IL-17A in the pathogenesis of periodontal diseases has received considerable attention. IL-17A has been demonstrated to have potent pro-osteoclastogenic effects and be a key driver of alveolar bone loss in periodontal diseases (Zenobia and Hajishengallis, 2015). Gingival crevicular fluid and saliva concentrations of ACh have previously been found to significantly correlate with levels of IL-17A in patients with periodontal disease (Apatzidou et al., 2018). Indeed, previous studies have suggested that there may be links between cholinergic systems and expression of IL-17A in mucosal tissues. The existence of $ChAT^{+}$ Th17 cells has been reported in the gut and this subpopulation of T cells has been demonstrated to release ACh, IL-17A, IL-22 and IFNγ in response to activation by primed dendritic cells (Dhawan et al., 2016). In addition, IL-17A has been demonstrated to induce the synthesis and release of ACh in bronchial epithelial cells (Montalbano et al., 2016) and non-neuronal ACh has also been demonstrated to play a role in promoting the differentiation of Th17 cells (Profita et al., 2014). Therefore, the clinical findings described in this manuscript could be attributed to an interplay between cholinergic systems and IL-17A biology within the oral mucosa which may

influence early implant osseointegration in patients with GAgP. However, further clinical studies reinforced by both *in vivo* and *in vitro* research is required to confirm this hypothesis.

Probing has limitations for assessment of implant success (Coli et al., 2017). In this study, MBL was determined on 2-D periapical radiographs. However, this may have caused underscoring of the real defect size as the image is a projection of the circumferential bone. An alternative approach could have been to use computed tomography (CT) as it can improve the resolution of anatomical structures allowing more accurate measurement. However, CT is currently not capable of accurately evaluating the implant circumferential bone level as it cannot accurately determine bone thickness. Moreover, it exposes the patient to a higher radiation dose than conventional 2-D imaging (De Bruyn et al., 2013). Therefore, radiographic evaluation continues to be the preferred method for evaluating implant health based on MBL (Cassetta et al., 2018).

A limitation of this study was the fact that smoking was not considered as an exclusion factor since one-third of the Turkish population >15 years are smokers (Ozer et al., 2018). Smoking impacts both cholinergic systems (Wessler et al., 2003, Wessler and Kirkpatrick, 2008) and IL-17 immunity (Qiu et al., 2017). In this study, there was no statistical difference between the smoking status of the patient cohorts. However, for future studies it would be more pertinent to recruit non-smoker patient cohorts if feasible.

Within the limits of the present study, it may be concluded that the GAgP has a significant impact on early healing phases in implant therapy which are associated with perturbations in cholinergic signalling mechanisms within the oral mucosa. These perturbations may have direct effects on bone metabolism via the presence of muscarinic and nicotinic receptors on osteoclasts and osteoblasts. In addition, the study provides more evidence for a relationship between cholinergic signalling and IL-17A which may demonstrate an indirect relationship between cholinergic signalling and early osseointegration events in implant placement. The current investigation therefore may provide a primer to further studies to investigate the therapeutic potential of targeting non-neuronal cholinergic mechanisms to treat peri-implantitis in patients with GAgP.

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## REFERENCES

ANITUA, E., ORIVE, G., AGUIRRE, J. J., ARDANZA, B. & ANDÍA, I. 2008. 5-year clinical experience with BTI dental implants: risk factors for implant failure. *J Clin Periodontol,* 35**,** 724-732.

APATZIDOU, D. A., ISKAS, A., KONSTANTINIDIS, A., ALGHAMDI, A. M., TUMELTY, M., LAPPIN, D. F. & NILE, C. J. 2018. Clinical associations between acetylcholine levels and cholinesterase activity in saliva and gingival crevicular fluid and periodontal diseases. *J Clin Periodontol,* 45**,** 1173-1183.

ARMITAGE, G. C. 1999. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol,* 4**,** 1-6.

ARREDONDO, J., CHERNYAVSKY, A. I. & GRANDO, S. A. 2007. SLURP-1 and -2 in normal, immortalized and malignant oral keratinocytes. *Life Sci,* 80**,** 2243-7.

ARREDONDO, J., HALL, L. L., NDOYE, A., CHERNYAVSKY, A. I., JOLKOVSKY, D. L. & GRANDO, S. A. 2003. Muscarinic acetylcholine receptors regulating cell cycle progression are expressed in human gingival keratinocytes. *J Periodontal Res,* 38**,** 79-89.

AWANG, R.A., LAPPIN, D.F., MACPHERSON, A., RIGGIO, M., ROBERTSON, D., HODGE, P., RAMAGE, G., CULSHAW, S., PRESHAW, P., TAYLOR, J., & NILE, C. 2014. Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity. *Inflamm Res*, 63(12), 1001-1012.

BUSER, D., JANNER, S.F., WITTNEBEN, J.G., BRÄGGER, U., RAMSEIER, C.A., SALVI, G.E. 2012. 10-year survival and success rates of 511 titanium implants with a sandblasted and acid-etched surface:

a retrospective study in 303 partially edentulous patients. *Clin Implant Dent Relat Res*,14(6), 839-851.

CASSETTA, M., DI GIORGIO, R. & BARBATO, E. 2018. Are Intraoral Radiographs Accurate in Determining the Peri-implant Marginal Bone Level? *Int J Oral Maxillofac Implants,* 33**,** 847-852.

COLI, P., CHRISTIAENS, V., SENNERBY, L. & BRUYN, H. 2017. Reliability of periodontal diagnostic tools for monitoring peri-implant health and disease. *Periodontol 2000,* 73**,** 203-217.

DE BRUYN, H., VANDEWEGHE, S., RUYFFELAERT, C., COSYN, J. & SENNERBY, L. 2013. Radiographic evaluation of modern oral implants with emphasis on crestal bone level and relevance to peri-implant health. *Periodontol 2000,* 62**,** 256-70.

DHAWAN, S., DE PALMA, G., WILLEMZE, R. A., HILBERS, F. W., VERSEIJDEN, C., LUYER, M. D., NUDING, S., WEHKAMP, J., SOUWER, Y., DE JONG, E. C., SEPPEN, J., VAN DEN WIJNGAARD, R. M., WEHNER, S., VERDU, E., BERCIK, P. & DE JONGE, W. J. 2016. Acetylcholine-producing T cells in the intestine regulate antimicrobial peptide expression and microbial diversity. *Am J Physiol Gastrointest Liver Physiol,* 311**,** G920-g933.

EN-NOSSE, M., HARTMANN, S., TRINKAUS, K., ALT, V., STIGLER, B., HEISS, C., KILIAN, O., SCHNETTLER, R. & LIPS, K. S. 2009. Expression of non-neuronal cholinergic system in osteoblast-like cells and its involvement in osteogenesis. *Cell Tissue Res,* 338**,** 203-15.

FISCHER, J., BOUADJAR, B., HEILIG, R., HUBER, M., LEFEVRE, C., JOBARD, F., MACARI, F., BAKIJA-KONSUO, A., AIT-BELKACEM, F., WEISSENBACH, J.,

LATHROP, M., HOHL, D. & PRUD'HOMME, J. F. 2001. Mutations in the gene encoding SLURP-1 in Mal de Meleda. *Hum Mol Genet,* 10**,** 875-80.

FUJII, T., MASHIMO, M., MORIWAKI, Y., MISAWA, H., ONO, S., HORIGUCHI, K. & KAWASHIMA, K. 2017. Expression and Function of the Cholinergic System in Immune Cells. *Front Immunol,* 8**,** 1085.

GATTI, C., GATTI, F., CHIAPASCO, M. & ESPOSITO, M. 2008. Outcome of dental implants in partially edentulous patients with and without a history of periodontitis: a 5-year interim analysis of a cohort study. *Eur J Oral Implantol,* 1**,** 45-51.

HAUPT, M., KAUSCHKE, V., SENDER, J., KAMPSCHULTE, M., KOVTUN, A., DURSELEN, L., HEISS, C. & LIPS, K. S. 2015. Bone status of adult female butyrylcholinesterase gene-deficient mice. *Int Immunopharmacol,* 29**,** 208-14.

HOOVER, D. B. 2017. Cholinergic modulation of the immune system presents new approaches for treating inflammation. *Pharmacol Ther,* 179**,** 1-16.

KARL, M. & ALBREKTSSON, T. 2017. Clinical Performance of Dental Implants with a Moderately Rough (TiUnite) Surface: A Meta-Analysis of Prospective Clinical Studies. *Int J Oral Maxillofac Implants,* 32**,** 717-734.

KAUSCHKE, V., KNEFFEL, M., FLOEL, W., HARTMANN, S., KAMPSCHULTE, M., DURSELEN, L., IGNATIUS, A., SCHNETTLER, R., HEISS, C. & LIPS, K. S. 2015. Bone status of acetylcholinesterase-knockout mice. *Int Immunopharmacol,* 29**,** 222-30.

KISTEMAKER, L. E., OENEMA, T. A., MEURS, H. & GOSENS, R. 2012. Regulation of airway inflammation and remodeling by muscarinic receptors: Perspectives on anticholinergic therapy in asthma and COPD. *Life Sci*.

LYUKMANOVA, E. N., SHULEPKO, M. A., KUDRYAVTSEV, D., BYCHKOV, M. L., KULBATSKII, D. S., KASHEVEROV, I. E., ASTAPOVA, M. V., FEOFANOV, A. V., THOMSEN, M. S., MIKKELSEN, J. D., SHENKAREV, Z. O., TSETLIN, V. I., DOLGIKH, D. A. & KIRPICHNIKOV, M. P. 2016. Human Secreted Ly-6/uPAR Related Protein-1 (SLURP-1) Is a Selective Allosteric Antagonist of alpha7 Nicotinic Acetylcholine Receptor. *PLoS One,* 11**,** e0149733.

MEI, D.M., ZHAO, B., XU, H., WANG, Y. 2017. Radiographic and clinical outcomes of rooted, platform switched, microthreaded implants with a sandblasted, large-grid, and acid-etched surface: A 5-year prospective study. Clin Implant Dent Relat Res, 19(6), 1074-1081.

MENGEL, R., BEHLE, M. & FLORES-DE-JACOBY, L. 2007. Osseointegrated implants in subjects treated for generalized aggressive periodontitis: 10-year results of a prospective, long-term cohort study. *J Periodontol,* 78**,** 2229-37.

MITO, K., SATO, Y., KOBAYASHI, T., MIYAMOTO, K., NITTA, E., IWAMA, A., MATSUMOTO, M., NAKAMURA, M., SATO, K. & MIYAMOTO, T. 2017. The nicotinic acetylcholine receptor alpha7 subunit is an essential negative regulator of bone mass. *Sci Rep,* 7**,** 45597.

MONJE, A., ALCOFORADO, G., PADIAL-MOLINA, M., SUAREZ, F., LIN, G. H. & WANG, H. L. 2014. Generalized aggressive periodontitis as a risk factor for dental implant failure: a systematic review and meta-analysis. *J Periodontol,* 85**,** 1398-407.

MONTALBANO, A. M., ALBANO, G. D., BONANNO, A., RICCOBONO, L., DI SANO, C., FERRARO, M., SIENA, L., ANZALONE, G., GAGLIARDO, R., PIEPER, M. P., GJOMARKAJ, M. & PROFITA, M. 2016. Autocrine Acetylcholine, Induced

by IL-17A via NFkappaB and ERK1/2 Pathway Activation, Promotes MUC5AC and IL-8 Synthesis in Bronchial Epithelial Cells. *Mediators Inflamm,* 2016**,** 9063842.

MORIWAKI, Y., TAKADA, K., NAGASAKI, T., KUBO, N., ISHII, T., KOSE, K., KAGEYAMA, T., TSUJI, S., KAWASHIMA, K. & MISAWA, H. 2015. IL-22/STAT3-Induced Increases in SLURP1 Expression within Psoriatic Lesions Exerts Antimicrobial Effects against Staphylococcus aureus. *PLoS One,* 10**,** e0140750.

MORIWAKI, Y., YOSHIKAWA, K., FUKUDA, H., FUJII, Y. X., MISAWA, H. & KAWASHIMA, K. 2007. Immune system expression of SLURP-1 and SLURP-2, two endogenous nicotinic acetylcholine receptor ligands. *Life Sci,* 80**,** 2365-8.

NAIK, R. S., LIU, W. & SAXENA, A. 2013. Development and validation of a simple assay for the determination of cholinesterase activity in whole blood of laboratory animals. *J Appl Toxicol,* 33**,** 290-300.

NGUYEN, V. T., HALL, L. L., GALLACHER, G., NDOYE, A., JOLKOVSKY, D. L., WEBBER, R. J., BUCHLI, R. & GRANDO, S. A. 2000. Choline acetyltransferase, acetylcholinesterase, and nicotinic acetylcholine receptors of human gingival and esophageal epithelia. *J Dent Res,* 79**,** 939-49.

NIZRI, E., WIRGUIN, I. & BRENNER, T. 2007. The role of cholinergic balance perturbation in neurological diseases. *Drug News Perspect,* 20**,** 421-9.

OFFENBACHER, S., BARROS, S. P. & BECK, J. D. 2008. Rethinking periodontal inflammation. *J Periodontol,* 79**,** 1577-84.

OZER, N., KILICKAP, M., TOKGOZOGLU, L., GOKSULUK, H., KARAASLAN, D., KAYIKCIOGLU, M., YILMAZ, M. B., BARCIN, C., ABACI, A. & SAHIN, M.

2018. Data on smoking in Turkey: Systematic review, meta-analysis and meta-regression of epidemiological studies on cardiovascular risk factors. *Turk Kardiyol Dern Ars,* 46**,** 602-612.

PAPAPANOU, P. N., SANZ, M., BUDUNELI, N., DIETRICH, T., FERES, M., FINE, D. H., FLEMMIG, T. F., GARCIA, R., GIANNOBILE, W. V., GRAZIANI, F., GREENWELL, H., HERRERA, D., KAO, R. T., KEBSCHULL, M., KINANE, D. F., KIRKWOOD, K. L., KOCHER, T., KORNMAN, K. S., KUMAR, P. S., LOOS, B. G., MACHTEI, E., MENG, H., MOMBELLI, A., NEEDLEMAN, I., OFFENBACHER, S., SEYMOUR, G. J., TELES, R. & TONETTI, M. S. 2018. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol,* 89 Suppl 1**,** S173-s182.

PJETURSSON, B. E., BRAGGER, U., LANG, N. P. & ZWAHLEN, M. 2007. Comparison of survival and complication rates of tooth-supported fixed dental prostheses (FDPs) and implant-supported FDPs and single crowns (SCs). *Clin Oral Implants Res,* 18 Suppl 3**,** 97-113.

POPE, C. N. & BRIMIJOIN, S. 2018. Cholinesterases and the fine line between poison and remedy. *Biochem Pharmacol,* 153**,** 205-216.

PROFITA, M., ALBANO, G. D., RICCOBONO, L., DI SANO, C., MONTALBANO, A. M., GAGLIARDO, R., ANZALONE, G., BONANNO, A., PIEPER, M. P. & GJOMARKAJ, M. 2014. Increased levels of Th17 cells are associated with non-neuronal acetylcholine in COPD patients. *Immunobiology,* 219**,** 392-401.

QIU, F., LIANG, C. L., LIU, H., ZENG, Y. Q., HOU, S., HUANG, S., LAI, X. & DAI, Z. 2017. Impacts of cigarette smoking on immune responsiveness: Up and down or upside down? *Oncotarget,* 8**,** 268-284.

RAJENDRAN, R., BORGHI, E., FALLENI, M., PERDONI, F., TOSI, D., LAPPIN, D. F., O'DONNELL, L., GREETHAM, D., RAMAGE, G. & NILE, C. 2015. Acetylcholine Protects against Candida albicans Infection by Inhibiting Biofilm Formation and Promoting Hemocyte Function in a Galleria mellonella Infection Model. *Eukaryot Cell,* 14**,** 834-44.

ROCCUZZO, M., BONINO, L., DALMASSO, P. & AGLIETTA, M. 2014. Long-term results of a three arms prospective cohort study on implants in periodontally compromised patients: 10-year data around sandblasted and acid-etched (SLA) surface. *Clin Oral Implants Res,* 25**,** 1105-12.

ROCCUZZO, M., DE ANGELIS, N., BONINO, L. & AGLIETTA, M. 2010. Ten-year results of a three-arm prospective cohort study on implants in periodontally compromised patients. Part 1: implant loss and radiographic bone loss. *Clin Oral Implants Res,* 21**,** 490-6.

ROSAS-BALLINA, M. & TRACEY, K. J. 2009. Cholinergic control of inflammation. *J Intern Med,* 265**,** 663-79.

SATO, T., ABE, T., CHIDA, D., NAKAMOTO, N., HORI, N., KOKABU, S., SAKATA, Y., TOMARU, Y., IWATA, T., USUI, M., AIKO, K. & YODA, T. 2010. Functional role of acetylcholine and the expression of cholinergic receptors and components in osteoblasts. *FEBS Lett,* 584**,** 817-24.

SWIERKOT, K., LOTTHOLZ, P., FLORES-DE-JACOBY, L. & MENGEL, R. 2012. Mucositis, peri-implantitis, implant success, and survival of implants in patients with treated generalized aggressive periodontitis: 3- to 16-year results of a prospective long-term cohort study. *J Periodontol,* 83**,** 1213-25.

TERNES, S., TRINKAUS, K., BERGEN, I., KNAACK, S., GELINSKY, M., KILIAN, O., HEISS, C. & LIPS, K. S. 2015. Impact of acetylcholine and nicotine on human osteoclastogenesis in vitro. *Int Immunopharmacol,* 29**,** 215-21.

VASILYEVA, N. A., LOKTYUSHOV, E. V., BYCHKOV, M. L., SHENKAREV, Z. O. & LYUKMANOVA, E. N. 2017. Three-Finger Proteins from the Ly6/uPAR Family: Functional Diversity within One Structural Motif. *Biochemistry (Mosc),* 82**,** 1702-1715.

WANG, L., ZHOU, Z., CHEN, Y., YUAN, S., DU, Y., JU, X., WU, L. & WANG, X. 2017. The Alpha 7 Nicotinic Acetylcholine Receptor of Deciduous Dental Pulp Stem Cells Regulates Osteoclastogenesis During Physiological Root Resorption. *Stem Cells Dev,* 26**,** 1186-1198.

WESSLER, I., KILBINGER, H., BITTINGER, F., UNGER, R. & KIRKPATRICK, C. J. 2003. The non-neuronal cholinergic system in humans: expression, function and pathophysiology. *Life Sci,* 72**,** 2055-61.

WESSLER, I. & KIRKPATRICK, C. J. 2008. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *Br J Pharmacol,* 154**,** 1558-71.

ZENOBIA, C. & HAJISHENGALLIS, G. 2015. Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol 2000,* 69**,** 142-59.

ZOHEIR, N., LAPPIN, D. F. & NILE, C. J. 2012. Acetylcholine and the alpha 7 nicotinic receptor: a potential therapeutic target for the treatment of periodontal disease? *Inflamm Res*.

## FIGURE LEGENDS

**Figure 1.** Box and whisker plots show the median and quartiles of the peri-implant crevicular fluid levels of **(A)** ACh, **(B)** AChE activity and **(C)** BChE activity around implants placed in the periodontium of healthy persons (Healthy implant) and patients with GAgP (GAgP implant) at baseline and 1-month post-surgery. * = P<0.05, ** = P<0.01 and *** P<0.001.

**Figure 2:** Box and whisker plots show the median and quartiles of the peri-implant crevicular fluid levels of **(A)** SLURP-1**, (B)** IL-17A, **(C)** IL-17E and **(D)** the IL-17A:IL-17E ratio around implants placed in the periodontium of healthy persons (Healthy implant) and patients with GAgP (GAgP implant) at baseline and 1-month post-surgery. * = P<0.05, ** = P<0.01 and *** P<0.001.

| | Male/ Female | Smoker/ Non-smoker | Age (years) | Marginal bone loss (1 month; mm) | Marginal bone loss (6 months; mm) |
|---|---|---|---|---|---|
| Healthy implants | 3 / 4 | 3 / 4 | 34.0<br>(33.0-35.5)<br>33.88 ± 3.99 | 0.00<br>(0.00-0.00)<br>0.00 ± 0.00 | 0.00<br>(0.00-0.00)<br>0.00 ± 0.00 |
| GAgP implants | 8 / 5 | 7 / 6 | 35.0<br>(33.0-38.0)<br>34.69 ± 4.63 | 0.00†<br>(0.00-0.16)<br>0.29 ± 0.63 | 0.001†‡<br>(0.00-0.65)<br>0.33 ± 0.48 |

For age and bone loss the data shown represents the median (interquartile range) and mean ± standard deviation. †Significantly different from healthy group. ‡Significantly different from 1 month value.

Kendal-Tau correlations between biomarkers in PICF at baseline and 1-month post implant placement. ** Correlation is significant at the 0.01 level (2-tailed).* Correlation is significant at the 0.05 level (2-tailed).

**A**

| | | AChE | BChE | IL-17A | IL-17E | IL17A:IL17E | SLURP-1 |
|---|---|---|---|---|---|---|---|
| ACh | Tau= | **0.389**** | 0.122 | **0.363**** | -0.09 | **0.395**** | 0.12 |
| | p= | **<0.001** | 0.264 | **0.001** | 0.407 | **<0.001** | 0.269 |
| AChE | Tau= | | 0.142 | **0.549**** | -0.001 | **0.435**** | **0.390**** |
| | p= | | 0.187 | **<0.001** | 0.992 | **<0.001** | **<0.001** |
| BChE | Tau= | | | 0.066 | **-0.275*** | 0.148 | -0.045 |
| | p= | | | 0.541 | **0.010** | 0.169 | 0.674 |
| IL-17A | Tau= | | | | 0.074 | **0.706**** | **0.441**** |
| | p= | | | | 0.486 | **<0.001** | **<0.001** |
| IL-17E | Tau= | | | | | **-0.219*** | **0.364**** |
| | p= | | | | | **0.040** | **0.001** |
| IL-17A:IL-17E | Tau= | | | | | | 0.189 |
| | p= | | | | | | 0.078 |

**B**

| | | AChE | BChE | IL-17A | IL-17E | IL-17A:IL-17E | SLURP-1 |
|---|---|---|---|---|---|---|---|
| ACh | Tau= | **0.674**** | 0.189 | **0.713**** | **0.507**** | **0.535**** | **0.584**** |
| | p= | **<0.001** | 0.084 | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| AChE | Tau= | | **0.369**** | **0.729**** | **0.568**** | **0.511**** | **0.388**** |
| | p= | | **0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| BChE | Tau= | | | **0.280*** | 0.147 | 0.143 | -0.029 |
| | p= | | | **0.011** | 0.18 | 0.192 | 0.794 |
| IL-17A | Tau= | | | | **0.448**** | **0.757**** | **0.472**** |
| | p= | | | | **<0.001** | **<0.001** | **<0.001** |
| IL-17E | Tau= | | | | | 0.205 | **0.560**** |
| | p= | | | | | 0.055 | **<0.001** |
| IL-17A:IL-17E | Tau= | | | | | | **0.344**** |
| | p= | | | | | | **0.001** |

Figure 1

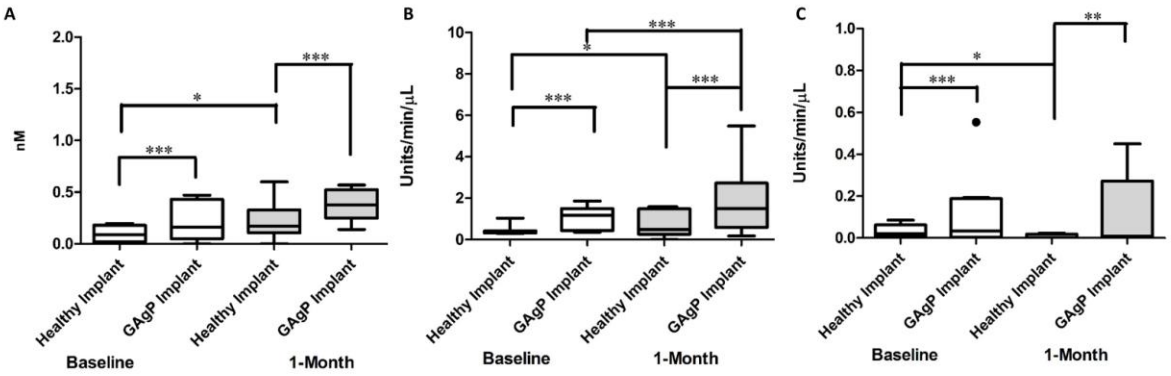


Figure 2