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Article type : Epidemiology (Cohort study or case-control study)

Clinical associations between acetylcholine levels and cholinesterase activity in saliva and gingival crevicular fluid and periodontal diseases

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RUNNING TITLE: Cholinergics and periodontal diseases

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jcpe.12989

KEYWORDS: Acetylcholine, Butyrylcholinesterase, IL-17, Periodontitis, Gingivitis

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Conflict of Interest

The authors declare that there are no conflicts of interest pertaining to this study.

Abstract

Aim

The oral mucosa possesses a non-neuronal cholinergic system. This study aimed to determine clinical evidence for a role of cholinergic mechanisms in the pathogenesis of periodontal diseases.

Materials and Methods

Fifty healthy participants, 52 patients with gingivitis and 49 with periodontitis were recruited. Full periodontal parameters were recorded and saliva and gingival crevicular fluid (GCF) collected. Levels of acetylcholine and inflammatory mediators were quantified using commercially available assay kits. Acetylcholinesterase and butyrylcholinesterase activity were measured using a published biochemical assay.

Results

Acetylcholine levels are significantly elevated in saliva and GCF, whereas GCF levels of butyrylcholinesterase activity are significantly decreased, in patients with periodontal diseases. Acetylcholine levels in saliva and GCF correlated positively with clinical markers of disease severity and with increased levels of IL-17A and IL-17F. In contrast, butyrylcholinesterase activity levels in GCF showed significant negative correlations with clinical markers of disease severity and IL-17A and IL-17F levels. None of the findings were due to smoking.

Conclusions

Elevated acetylcholine levels and reduced butyrylcholinesterase activity are clinically associated with periodontal diseases and elevated levels of IL-17A and IL-17F. Therefore, non-neuronal cholinergic mechanisms may influence IL-17 biology and the aetiopathogenesis of periodontal diseases and therefore are possible therapeutic targets.

Clinical Relevance

Scientific rationale for study: The oral mucosa possesses non-neuronal cholinergic mechanisms. Mucosal cholinergic mechanisms are associated with inflammatory diseases and important for maintenance of barrier function and modulating immune responses. The role of cholinergic mechanisms in the aetiopathogenesis of periodontal diseases is unknown.

Principal findings: Perturbations in salivary and gingival crevicular fluid levels of acetylcholine and butyrylcholinesterase activity are associated with severity of periodontal diseases and levels of IL-17A and IL-17F.

Practical implications: The data demonstrates the need for further study into the role of cholinergic mechanisms in periodontal diseases and provide evidence that cholinergic mechanisms may be therapeutic targets.

Introduction

The role of non-neuronal acetylcholine (ACh) as a cytotransmitter is well established (Wessler and Kirkpatrick, 2008). Choline acetyltransferase (ChAT) is responsible for the synthesis of ACh from acetyl coenzyme A and choline. ACh signalling is mediated by muscarinic and nicotinic receptors. Both receptor subtypes are found to be expressed by non-neuronal cells (Albuquerque et al., 2009).

ACh is extremely labile due to the action of esterases (Klapproth et al., 1997). In humans acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are responsible for the hydrolysis of acetylcholine to acetic acid and free choline (Grando et al., 1993).

The oral mucosa can be definedas having a non-neuronal cholinergic system (Nguyen et al., 2000, Arredondo et al., 2003, Macpherson et al., 2014, Zoheir et al., 2012) due to the fact it expresses ChAT, acetylcholine receptors and cholinesterases (Wessler and Kirkpatrick, 2008, Rana et al., 2010). Roles for non-neuronal ACh within mucosal tissues include; cell proliferation and differentiation, cytoskeletal organization, cell–cell contact, maintenance of effective barrier functions, controlling ion and water movements and modulation of immune responses (Wessler and Kirkpatrick, 2008, Macpherson et al., 2014).

The role of ACh as an immunomodulatory neuroendocrine mediator first came to prominence with the discovery of the 'cholinergic anti-inflammatory pathway' (Borovikova et al., 2000). In this pathway, signals transmitted by parasympathetic nervous system induce the activation and release of ACh by a novel choline acetyltransferase positive ($ChAT^+$) T cell subset which subsequently down regulates proinflammatory cytokine expression by localised macrophages in the spleen via the α7 nicotinic receptor (α7nAChR) (Rosas-Ballina et al., 2011, Martelli et al., 2016). $ChAT^+$ T cells can also emigrate to peripheral sites and mediate parasympathetic anti-inflammatory effects within mucosal tissues (Dhawan et al., 2016). $ChAT^+$ B cell populations in the intestinal mucosa have also been found to release ACh upon antigen stimulation which in turn modulates neutrophil recruitment (Reardon et al., 2013). Furthermore, oral keratinocytes have been found to release ACh in response to stimulation by *Porphyromonas gingivalis* and the α7nAChR on oral keratinocytes has been demonstrated to downregulate *P. gingivalis* induced expression of IL-8 (CXCL8) (Macpherson et al., 2014). The evidence therefore, suggests that ACh can modulate innate immune responses within the oral mucosa and subsequently the pathogenesis of periodontal diseases (Zoheir et al., 2012).

In vivo models have demonstrated a role for cholinergic systems in the pathogenesis of inflammatory diseases (Hoover, 2017). However, there is a fundamental lack of clinical studies associating ACh or cholinesterase activity with disease outcomes. ACh levels have been associated with diseases of impaired cognitive function (Jia et al., 2004, Yamada et al., 1996) and, with respect to the oral cavity, one study demonstrated that ACh levels in gingival tissue are elevated during inflammation (Rajeswari and Satyanarayana, 1990). Furthermore, cholinesterase activity has been measured in saliva to determine its use as a diagnostic marker of Alzheimer's disease (Sayer et al., 2004). To date, however, no studies have investigated whether cholinergic mediators are associated with periodontal diseases. Therefore, the aim of this study was to determine if ACh and esterase activity could be

measured in saliva and GCF and determine their relationship with levels of immunoregulatory mediators and clinical outcomes of periodontal diseases.

Materials and Methods

Ethics

The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki, and was approved by the Ethics Committee of the School of Dentistry, Aristotle University of Thessaloniki (AUTh), Greece (9/28.01.2014).

Study Population

A total of 151 individuals were recruited from either patients attending the Lab of Preventive Dentistry, Periodontology and Implant Biology for periodontal treatment, or from faculty members and postgraduate students, at AUTh. Probing pocket depth (PPD); clinical attachment levels (CAL); bleeding on probing (BOP) and plaque indices (PI) were determined at six sites per tooth by a calibrated single examiner (AI). PPD and CAL were measured using an electronic constant-force (15g pressure) probe (FP32, software version 9; Florida Probe Corporation©, Florida, USA) to the nearest 0.2 mm.

Inclusion criteria: (i) Periodontitis patients (n=49) having at least two sites per quadrant with PPD and CAL >5mm and radiographic evidence of bone loss; (ii) Gingivitis patients (n=52) presenting with BOP at >30% of sites, PPD <4mm and no radiographic evidence of bone loss; and (iii) healthy subjects (n=50) having <10% sites with BOP, and no sites with PPD >3mm and CAL >4mm.

Exclusion criteria: presence of <18 teeth (excluding third molars), periodontal treatment within the past 12 months, history of systemic disease, pregnancy or lactation, and prophylactic antibiotics, cholinergic therapies or anti-inflammatory medications within the last 3 months.

Saliva and GCF collection

Unstimulated whole saliva was collected in the morning preceding all other assessments following an overnight fast during which subjects were requested not to drink (except water) or chew gum (Navazesh, 1993). The saliva was centrifuged at 4000 rpm for 20 minutes at 20 ^{o}C to remove debris and the supernatant stored at -80 ^{o}C until required for analysis.

GCF was collected using Periopaper (OraFlow Inc., Smithtown, NY, USA) over 30 s as previously described (Awang et al., 2014) from four sites per patient that were non-adjacent, had no endodontic involvement and were the deepest in each quadrant (sPPD, sCAL both >5 mm for periodontitis cases). The strips were pooled and the GCF eluted into 1 ml of phosphate buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) and stored at -80 ^{o}C until further analysis.

Quantification of Acetylcholine levels in Saliva and gingival crevicular fluid

Levels of ACh were quantified using the Acetylcholine Assay Kit (Abcam, Cambridge, UK) in colorimetric assay mode as previously described (Reale et al., 2012). Briefly, to determine total choline, 50 µl of saliva or GCF eluate was mixed with 50 µL of a reaction solution containing: choline assay buffer, choline probe, enzyme mix and acetylcholinesterase (AChE). Free choline was determined as above except for omitting the AChE. A standard curve was generated using a supplied choline standard. Each sample was analysed in triplicate. The reactions were allowed to proceed for 30 minutes at room temperature in the

dark and colorimetric changes at 570 nm read on a plate reader (BMG-Labtech, Ortenberg, Germany). Acetylcholine levels were determined using the following equation: Acetylcholine = Total choline – Free choline.

Measurement of cholinesterase activity in saliva and GCF

Analysis of AChE and BChE activity levels was performed as described by Naik *et al* (Naik et al., 2013). Briefly, 10 µl of saliva or 20 µl of GCF was added to 50 mM sodium phosphate buffer, pH 8.0; 1.3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 20 mM ethopropazine or 500 nM huperzine A to a final volume of 250 µl. Following incubation at 22 ^{o}C for 20 min, 50 µl of 6 mM Acetylthiocholine (ATC) or Butyrylthiocholine (BTC) was added and rates of ATC/BTC hydrolysis were obtained by reading the plates at 412 nm for 10 min. The rates of substrate hydrolysis were corrected for a 1 cm path length, dilution factor and an extinction coefficient of 14.15 x 10^3 M/cm for DTNB to obtain activity in units per ml (Eyer et al., 2003). One unit (U) of enzyme activity is defined as the hydrolysis of 1 µM of substrate in 1 minute.

Determination of cytokine and soluble receptor levels in saliva and GCF

Levels of IL-4, IL-6, TNF, IL-17A, IL-17F and IL-17E (all Peprotech, UK), IL-17A/F and IL-23R, (both R&D Systems, UK), TGF-β and IL-23 (both Mabtech, Sweden) were determined using commercially available ELISA kits as per the manufacturer's instructions. The limits of detection were determined as two mean standard deviations higher than the mean baseline from six replicate standard curves: IL-4, IL-6, TNF, IL-17A and IL-17F = 1.9 pg/ml; IL-17E = 3.8 pg/ml; IL-17A/F, IL-23R, TGF-β and IL-23 = 4.2 pg/ml. The IL-17A:IL-17E ratio was determined as previously described (Awang et al., 2014).

Statistical analysis

A statistical power calculation was performed based on the assumption that the data were normally distributed (or log normally distributed) and that Central Limit Theorem applied. After adjusting α to 0.05/3 for the comparison of 3 groups and using effect sizes of 0.7 with parametric statistical tests the average numbers per group required to exceed 80% statistical power were n=45.

Statistical analysis was performed using an ANOVA and a multivariate analysis in a General Linear Model. Adjustments were made for potential confounding factors such as age and smoking using least-square regression analysis. The figures show unweighted results; however, the statistical differences indicated reflect the results of the weighted analyses.

Associations between data were assessed using a Pearson correlation coefficient. All tests were performed at α=0.05 significance level. Partial correlation analysis adjusting for age was used to explore potential associations. The large variation in cigarette usage meant partial correlation analysis correcting for smoking was statistically impossible. Therefore, analysis was also performed separately on the never smoker subjects to ascertain which associations remained after removal of smoking completely as a confounding factor.

Results

Study Population

Table 1 shows the proportion of subjects who smoked was significantly greater in the gingivitis group and the periodontitis group compared to the healthy group (both $p<0.05$). In addition, the cigarettes/day*yr was significantly greater in the periodontitis group compared

to the healthy group ($p<0.05$). The age of the subjects was also significantly higher in the periodontitis group compared to the healthy group (both $p<0.001$).

All clinical parameters measured, after weighting for age and smoking status, were significantly greater in the gingivitis group and periodontitis group compared to the healthy group (all $p<0.001$). All clinical parameters measured except PI and sPI were significantly greater in the periodontitis group compared to the gingivitis group (all $p<0.001$).

Acetylcholine levels and cholinesterase activity in saliva and gingival crevicular fluid

Figure 1A shows that salivary levels of ACh were significantly elevated in the periodontitis group compared to the healthy group ($p<0.01$) and the gingivitis group ($p<0.05$). Figure 1B shows that GCF levels of ACh were significantly elevated in the periodontitis group compared to the healthy group ($p<0.001$) and the gingivitis group ($p<0.001$).

No significant inter-group differences were observed with respect to salivary (Figure 2A) and GCF (Figure 2B) AChE activity and salivary BChE activity (Figure 2C). However, GCF BChE activity was significantly decreased in the periodontitis ($p<0.01$) and gingivitis group ($p<0.05$) compared to the healthy group (Figure 2D).

Cytokine and soluble receptor levels in saliva and GCF

Table 2A shows that salivary levels of IL-17A, IL-17A/F, IL-4 and the IL-17A:IL-17E ratio were significantly increased in the periodontitis group compared with the healthy group (all $p<0.01$). Furthermore, salivary levels of IL-17A, IL-17A/F, and the IL-17A:IL-17E ratio were significantly increased in the periodontitis group compared to the gingivitis group (all

p<0.05).

Table 2B shows that GCF levels of IL-17A, IL-17F, IL-17A/F and the IL-17A:IL-17E ratio were all significantly increased in the gingivitis group compared to the healthy group (all p<0.05). GCF levels of IL-17A, IL-17F, IL-6 and the IL-17A:IL-17E ratio were all significantly increased in the periodontitis group compared to the healthy group (all p<0.05). In addition, GCF levels of TGF-β are significantly decreased in the periodontitis group compared to the healthy group (p<0.05). Furthermore, GCF levels of IL-17A, IL-17F and IL-6 were all significantly increased in the periodontitis group and IL-17A/F levels significantly decreased compared to the gingivitis group (all p<0.05).

Correlations between acetylcholine levels and esterase activity in saliva and GCF and clinical parameters

Table 3A shows the major correlations between salivary levels of ACh, esterase activity and clinical parameters. The key findings are as follows: Salivary levels of ACh had weak significant positive correlations with all clinical indices when all participants (n=151; all p<0.05) and never smokers (n=94; all p<0.05) were considered. Saliva AChE activity showed a moderate to strong significant positive correlation with BChE activity in all participants (p<0.001) and never smokers (p<0.001). Saliva BChE activity also showed a weak significant positive correlation with mean PD ≥5 mm in all participants (p<0.05).

Table 3B shows the major correlations between GCF levels of ACh, esterase activity and clinical parameters. The key findings are as follows: GCF levels of ACh had a weak significant positive correlation with BChE only when all participants were considered (all p<0.01). GCF levels of ACh showed moderate to strong significant positive correlations with

sBOP, sPI, sPPD and sCAL when all participants (n=151; all $p<0.001$) and never smokers (n=94; all $p<0.001$) were considered. A significant weak negative correlation between BChE activity and sBOP, sPPD and sCAL was also determined in all participants (all $p<0.01$) and never smokers (all $p<0.05$).

Correlations between acetylcholine levels and esterase activity in saliva and GCF and levels of selected inflammatory mediators

Table 4A shows the major correlations between salivary levels of ACh, esterase activity and levels of selected inflammatory mediators. The key finding was that salivary levels of ACh had weak significant positive correlations with IL-17A and IL-17A/F when all participants were considered (both $p<0.05$) but not in never smokers.

Table 4B shows the major correlations between GCF levels of ACh, esterase activity and levels of selected inflammatory mediators. The key findings are as follows:

GCF levels of ACh had moderate to strong significant positive correlations with IL-17A and IL-17F in all participants (both $p<0.001$) and never smokers only ($p<0.001$). ACh levels had a significant weak positive correlation with the IL-17A:IL-17E ratio and levels of IL-6 only when all participants were considered (both $p<0.01$). GCF BChE activity showed significant weak negative correlations with levels of IL-17A and IL-17F in all participants (both $p<0.01$) and never smokers (both $p<0.05$). GCF BChE activity showed significant weak negative correlations with IL-17A/F in never smokers only ($p<0.01$). GCF BChE activity showed significant weak negative correlations with the IL-17A:IL-17E ratio and levels of IL-6 in all participants only (both $p<0.05$).

Discussion

The data in this manuscript provide clinical evidence that cholinergic mechanisms may play a role in the pathogenesis of periodontal diseases. Significantly elevated levels of ACh are found in saliva and GCF of patients with gingivitis and periodontitis. Furthermore, GCF levels of ACh showed significant positive correlations with clinical parameters and levels of IL-17A and IL-17F. In contrast, GCF BChE activity is significantly decreased in patients with gingivitis and periodontitis and showed significant negative correlations with clinical parameters and levels of IL-17A and IL-17F.

A limitation of this study was the fact that smoking was not considered as an exclusion factor. Evidence suggests that smoking can impact on non-neuronal cholinergic mechanisms (Wessler and Kirkpatrick, 2008, Wessler et al., 2003, Beckmann and Lips, 2013). Furthermore, smoking has profound effects on the immune system (Qiu et al., 2017) and more specifically on IL-17 biology. Cigarette smoke extract and nicotine have been shown to downregulate Th17 responses (Peruzzo et al., 2016). Furthermore, IL-17 levels in GCF of gingivitis and periodontitis patients have been shown to be decreased in smokers compared to non-smokers (Peruzzo et al., 2016, Buduneli et al., 2009). However, in this study, weighted analysis to correct for smoking had little effects on the statistical significance of the perturbations in ACh levels and cholinesterase activity in biological fluids reported in periodontal diseases. Furthermore, key correlations between ACh levels, cholinesterase activity, clinical parameters and levels of inflammatory mediators remained significant when only never smokers were considered. Therefore, the evidence suggests that smoking status had little impact on the major findings reported.

The role of ACh as an anti-inflammatory neuroendocrine mediator has received a great deal of attention since the discovery of the cholinergic anti-inflammatory pathway (Borovikova et al., 2000). It is now well established that non-neuronal ACh, acting via the α7nAChR, can also negatively regulate proinflammatory mediator expression by both professional and non-professional immune cells (Yoshikawa et al., 2006, de Jonge et al., 2005, Macpherson et al., 2014). However, evidence from *in vivo* and *in vitro* infection models suggests that it is too simplistic to state that within complex mucosal tissues ACh acts purely in an anti-inflammatory capacity (Giebelen et al., 2008, Giebelen et al., 2009, Rajendran et al., 2015). There is indeed evidence to support a role for ACh in promoting neutrophil recruitment, activation and neutrophil extracellular trap (NET) formation (Ruhnau et al., 2014, Xu et al., 2008, Rajendran et al., 2015, Giebelen et al., 2008, Carmona-Rivera et al., 2017). A plausible explanation for these differential findings is the fact that immune cells possess a plethora of nicotinic and muscarinic receptors which may differentially regulate immune responses. For example muscarinic receptors, in particular the M3 subtype, have been found to promote innate immune responses both *in vitro* (Kawashima and Fujii, 2004, Buhling et al., 2007) and *in vivo* (McLean et al., 2016, Kistemaker et al., 2013).

It is now evident that numerous immune cells express ChAT including T cells, B cells, dendritic cells and macrophages (Fujii et al., 2017). Evidence suggests that T and B cells can acquire the ability to produce ACh in the mucosa associated lymphoid tissue (MALT) prior to emigration to peripheral sites (Reardon et al., 2013). Interestingly, ChAT expression by B and T cells is hypothesised to be dependent on the establishment of the host microbiota and occurs in response to Toll-Like Receptor (TLR) agonists (Friswell et al., 2010, Reardon et al., 2013). Furthermore, evidence suggests that the ability of immune cells to express ChAT is in fact transient and not lineage specific (Reardon et al., 2013). Immune cells, like neurons, have been shown to synthesise and store ACh. In T and B cells the actual release of ACh has been found to be regulated by Norepinephrine and

Cholecystokinin, respectively (Rosas-Ballina et al., 2011, Reardon et al., 2013). Furthermore, $ChAT^+$ T cells and $ChAT^+$ B cells have been shown to have differential immunomodulatory functions (Reardon et al., 2013). Moreover, there is now evidence for $ChAT^+$ T cells with characteristics of both Treg (Reardon et al., 2013) and Th17 cells (Dhawan et al., 2016). Interestingly, $ChAT^+$ Th17 cells have been identified in payer's patches and the lamina propria of intestinal tissue and are activated by dendritic cells, in response to stimulation by adrenergic neurotransmitters, to release ACh, IL-17A, IL-22 and IFNγ which in turn drives antimicrobial peptide expression by the intestinal epithelia and results in alterations in the diversity and richness of the gut microflora (Dhawan et al., 2016). Therefore ACh may not only regulate the host response to complex multispecies biofilms but also influence biofilm composition directly therefore having much more complex roles in biofilm driven inflammatory diseases such as periodontal diseases.

Extracellular ACh is extremely labile due to the action of esterases (Wessler et al. 2003). The amount of available ACh within tissues is therefore modulated by a careful balance of synthesis and degradation. In humans the principle cholinesterases are AChE and BChE (Wessler and Kirkpatrick, 2008). Although AChE is thought to be principally membrane bound there is evidence that within some tissues, including the neuromuscular junction and brain, a soluble form exists (Hicks et al., 2011). In contrast, the presence of both membrane bound and soluble forms of BChE in humans is well documented (Darvesh et al., 2003). Evidence has shown that perturbations in BChE activity are associated with diseases with an inflammatory component (Darvesh et al., 2003). For example, serum BChE activity levels are reduced in sterile inflammatory responses to surgery and in response to traumatic injury (Zivkovic et al., 2017, Zivkovic et al., 2016). Moreover, genetic variations in BChE have been associated with autoimmune diseases such as arthritis and multiple sclerosis (Shahmohamadnejad et al., 2015, Reale et al., 2018).

Based on current findings, and previous data, there is strong evidence for a role of dysregulated cholinergic mechanisms in the pathogenesis of periodontal disease. The strong correlations between elevated levels of ACh and proinflammatory members of the IL-17 family of cytokines at specific sites open up the possibility that ChAT+ Th17 cells may play a role in modulating the oral microbiota, as observed in the intestinal mucosa (Dhawan et al., 2016), and thus contribute to the aetiopathogeneis of periodontal disease. Furthermore, the elevated levels of ACh in GCF at inflamed sites may in part due to decreased BChE activity which in turn may be due to polymorphisms in the *BChE* gene. However, further *in vitro* and *in vivo* studies are required to unravel the mechanistics behind these clinical findings. That said, the data described herein open up the potential that therapeutic modalities targeting cholinergic mechanisms may be of use for the treatment of periodontal diseases.

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Figure Legends

Figure 1: Saliva and gingival crevicular fluid levels of acetylcholine are upregulated in periodontal diseases. Comparison of saliva (A) and gingival crevicular fluid (B) levels of ACh between healthy subjects, subjects diagnosed with gingivitis and subjects diagnosed with periodontitis. The statistical significances shown are following adjustment for age and smoking. *$p<0.05$, **$p<0.01$,***$p<0.01$.

Figure 2: Gingival crevicular fluid butyrylcholinesterase activity is decreased in periodontal diseases. Comparison of saliva (A) and gingival crevicular fluid (B) levels of AChE activity between healthy subjects, subjects diagnosed with gingivitis and subjects diagnosed with periodontitis. Comparison of saliva (C) and gingival crevicular fluid (D) levels of BChE activity between healthy subjects, subjects diagnosed with gingivitis and subjects diagnosed with periodontitis. The statistical significances shown are following adjustment for age and smoking. *$p<0.05$, **$p<0.01$,***$p<0.01$.

Table 1

	Healthy (n=50)		Gingivitis (n=52)		Periodontitis (n=49)	
Female	68 % (n=34)		46 % (n=24)		47 % (n=23)	
Smokers	18 % (n=9)		**22 % (n=12)***		**49 % (n=24)***	
Former smokers	0 % (n=0)		10 % (n=5)		14 % (n=7)	
Never smokers	82 % (n=41)		68 % (n=35)		37 % (n=18)	
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)
Cigs/days*yrs	*13335*	*(-16975 – 43646)*	*20729*	*(-8982 - 50439)*	***115824***	***(85514 - 146135)***†††***
AGE	*32.5*	*(28.9 - 36.1)*	*35.5*	*(32.0 -39.0)*	***50.2***	***(46.7 - 53.8)***†††***
BOP	0.13	(0.09 – 0.17)	**0.37**	**(0.31 – 0.44)*****	**0.53**	**(0.38 – 0.68)***†††***
PI	0.24	(0.19 – 0.30)	**0.58**	**(0.49 – 0.68)*****	**0.59**	**(0.38 – 0.84)*****
PPD	1.79	(1.47 – 1.60)	1.80	(1.70 – 2.00)	**2.96**	**(2.74 – 3.20)***†††***
CAL	1.59	(1.50 – 1.70)	1.80	(1.60 – 2.00)	**3.60**	**(3.26 – 3.95)***†††***
PPD ≥ 5 mm	0.15	(-0 64 – 0.95)	1.00	(-0.33 – 2.34)	**11.9**	**(0.93 – 13.75)***†††***
PPD > 7 mm	0.00	(-0.20 – 0.20)	0.19	(-0.15 – 0.52)	**2.13**	**(0.16 – 2.60)***†††***
PPD 5-7 mm	0.15	(-0.49 – 0.79)	0.81	(-0.27 – 1.90)	**9.71**	**(8.17 – 11.24)***†††***
mean PPD ≥ 5 mm	0.00	(-0.01 – 0.01)	0.01	(-0 .01– 0.02)	**0.09**	**0.07 – 0.10)***†††***
mean PPD > 7 mm	0.00	(0.00 – 0.00)	0.00	(0 00 – 0.00)	**0.02**	**(0.01 – 0.05)***†††***
mean PPD 5-7 mm	0.00	(-0.01 – 0.01)	0.01	(-0.01 – 0.02)	**0.07**	**(1.79 – 2.20)***†††***

sBOP	0.11	(0.05 – 0.17)	**0.47**	**(0.37 – 0.58)*** **	**0.72**	**(0.06 – 0.08)*** †††**
sPI	0.18	(0.12 – 0.25)	**0.51**	**(0.50 – 0.72)*** **	**0.51**	**(0.34 – 0.68)*** **
sPPD	1.54	(1.40 – 1.68)	1.86	(1.68 – 2.14)	**5.14**	**(4.80 – 5.48)*** †††**
sCAL	1.7	(1.4 – 1.8)	1.87	(1.54 – 2.21)	**5.64**	**(5.30 – 5.99)*** †††**

*Significantly different from the healthy group, † significantly different between the periodontitis and gingivitis group. BOP = full mouth bleeding on probing, PI = full mouth plaque index, PPD = full mouth pocket depth, CAL = full mouth attachment loss, PPD ≥ 5 mm = number of sites in mouth with a PD equal to or greater than 5 mm, PPD ≥ 7 mm = number of sites in mouth with a PD equal to or greater than 7 mm, PPD 5-7 mm = number of sites in mouth with a PD between 5 and 7 mm. s= clinical parameters at specific GCF sampling site. Data in italics is not weighted. The statistical analysis of the clinical indices was weighted to correct for age and smoking.

Table 2

	Healthy (n=50)		Gingivitis (n=52)		Periodontitis (n=49)	
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)
A. Saliva						
IL-17A	229.7	(111.0-348.4)	78.4	(-131.2-288.0)	**924.3**	**(591.0-1257.6)*†††**
IL-17F	152.2	(48.7-255.7)	104.2	(-78.4-286.9)	170.2	(-120.3-460.7)
IL-17A/F	157.3	(82.5-232.1)	86.0	(-46.1-218.0)	**520.7**	**(310.7-730.6) *†††**
IL-17E	198.4	(133.9-262.9)	142.1	(28.3-255.9)	111.2	(-69.8-292.2)
IL-17A/IL-17E	0.337	(0.135-0.539)	0.169	(-0.188-0.526)	**2.31**	**(1.71-2.82) *†††**
IL-4	129.6	(87.3-172.0)	127.2	(52.5-202)	**151.8**	**(33.0-270.7)***
IL-6	369.2	(111.7-626.7)	875.7	(421.1-1330.3)	1252.9	(529.9-1975.9)
TNF	540.0	(336.1-743.9)	78.7	(-281.2-438.7)	499.5	(-73.0-1071.9)
TGF-β	235.9	(174.1-297.7)	244.5	(135.4-353.6)	112.3	(-61.2-285.9)
IL-23	112.7	(36.9-188.6)	80.7	(-53.173-214.5)	134.3	(-78.6-347.1)
IL-23R	48.2	(33.5-62.9)	47.8	(21.9-73.8)	39.5	(-1.8-80.8)
B. GCF						
IL-17A	20.5	(9.5-31.5)	**51.1**	**(31.7-70.5)***	**114.7**	**(83.8-145.6)**†**
IL-17F	15.8	(8.2-23.4)	**47.4**	**(34.0-60.8)***	**133.4**	**(112.1-154.7)**†**
IL-17A/F	9.5	(-1.9-20.9)	**60.1**	**(40.0-80.2)***	**25.6**	**(-6.3-57.6)†**

IL-17E	24.4	(17.3-31.4)	15.6	(3.1-28.0)	18.2	(-1.6-37.9)
IL-17A/IL-17E	1.1	(-1.2-3.4)	**10.0**	**(6.0-14.1)****	**9.1**	**(3.6-14.5)***
IL-4	2.7	(2.2-3.2)	3.8	(2.9-4.7)	5.3	(3.9-6.8)*
IL-6	7.8	(4.6-11.0)	12.9	(7.3-18.6)	**30.0**	**(20.9-39.0)*†**
TNF	17.3	(14.0-20.5)	16.0	(10.2-21.7)	19.3	(10.1-28.4)
TGF-β	32.2	(30.0-34.5)	23.0	(19.0-27.0)	**20.5**	**(14.2-26.9)***
IL-23	6.5	(3.0-10.1)	6.2	(-0.1-12.5)	5.5	(-4.5-15.4)
IL-23R	13.0	(6.7-19.2)	26.6	(15.5-37.7)	9.8	(-7.9-27.4)

*Significantly different from the healthy group, † significant difference between periodontitis and gingivitis group. The statistical analysis was weighted to correct for age and smoking.

Table 3

A. Saliva

		AChE		BChE		BOP		PI		PPD		CAL		mean PPD ≥5mm	
		AP	NS	AP	NS	AP	NS	AP	NS	AP	NS	AP	NS	AP	NS
ACh	*r*	-.072	-.020	.015	.030	**.165***	**.235***	**.196***	**.236***	**.289*****	**.263****	**.295*****	**.271****	**.273*****	**.203****
(nmol/ml)	*p*	.387	.844	.853	.773	**.043**	**.022**	**.016**	**.022**	**<.001**	**.009**	**<.001**	**.008**	**.001**	**.049**
	n	151	94	151	94	**151**	**94**	**151**	**94**	**151**	**94**	**151**	**94**	**151**	**94**
AChE	*r*			**.481*****	**.540*****	**.173***	.179	**.216****	**.204***	.158	**.222****	.137	.187	**.207***	**.350*****
(Units)	*p*			**<.001**	**<.001**	**.036**	.085	**.008**	**.049**	.055	**.032**	.096	.073	**.012**	**.001**
	n			**151**	**94**	**151**	94	**151**	**94**	151	**94**	151	94	**151**	**94**
BChE	*r*					.152	.165	.118	.088	.120	.122	.095	.092	**.168***	.162
(Units)	*p*					.065	.112	.152	.400	.146	.245	.253	.873	**.041**	.121
	n					151	94	151	94	151	94	151	94	**151**	94

B. GCF

		AChE		BChE		sBOP		sPI		sPPD		sCAL	
		All	NS	All	NS	All	NS	All	NS	All	NS	All	NS
ACh	*r*	.151	.122	**-.215****	-.184	**.407*****	**.459*****	**.306*****	**.322*****	**.545*****	**.560*****	**.560*****	**.560*****
(nmol/ml)	*p*	.064	.239	**.008**	.082	**<.001**	**<.001**	**<.001**	**.001**	**<.001**	**<.001**	**<.001**	**<.001**
	n	151	94	**151**	94	**151**	**94**	**151**	**94**	**151**	**94**	**151**	**94**
AChE	*r*			**.270*****	**.286****	**.256****	.201	**.208***	**.222***	.139	.120	.149	.116
(Units)	*p*			**.001**	**.005**	**.002**	.055	**.011**	**.031**	.090	.246	.069	.249
	n			**151**	**94**	**151**	94	**151**	**94**	151	94	151	94
BChE	*r*					**-.272*****	**-.278****	-.144	-.048	**-.256****	**-.228***	**-.257****	**-.219***
(Units)	*p*					**.001**	**.006**	.079	.641	**.002**	**.027**	**.002**	**.033**
	n					**151**	**94**	151	94	**151**	**94**	**151**	**94**

*Correlation is significant at the 0.05 level, ** at the 0.01 level, *** at the 0.001 level (all 2-tailed and shown in bold text). ACh = Acetylcholine, AChE = Acetylcholinesterase, BChE = Butyrlcholinesterase, AP = All participants, NS = Never smokers only.

Table 4

A. Saliva

		IL-17A		IL-17F		IL-17A/F		IL-17A:IL-17E		IL-4		IL-6		TNF	
		AP	NS	AP	NS	AP	NS	AP	NS	AP	NS	AP	NS	AP	NS
ACh	*r*	**.201***	.082	-.017	.176	**.224****	.107	-.057	.063	.000	.105	.018	.045	-.048	.191
(nmol/ml)	*p*	**.014**	.433	.838	.088	**.006**	.301	.497	.544	.996	.312	.828	.663	.588	.064
	n	**151**	94	151	94	**151**	94	151	94	151	94	151	94	151	94
AChE	*r*	.064	.084	-.055	-.045	.058	.115	.003	-.049	-.074	.007	-.072	-.068	-.082	-.019
(Units)	*p*	.442	.424	.505	.665	.487	.271	.972	.640	.370	.951	.384	.518	.322	.862
	n	151	94	151	94	151	94	151	94	151	94	151	94	151	94
BChE	*r*	-.052	-.167	-.002	-.064	-.038	-.041	-.043	-.089	-.150	**-.267****	-.078	-.065	**-.225***	**-.237***
(Units)	*p*	.530	.110	.984	.554	.644	.690	.617	.396	.069	**.008**	.349	.537	**.006**	**.022**
	n	151	94	151	94	151	94	151	94	151	**94**	151	94	**151**	**94**

B. GCF

		IL-17A		IL-17F		IL-17A/F		IL-17A:IL-17E		IL-4		IL-6		IL-23	
		AP	NS	AP	NS	AP	NS	AP	NS	AP	NS	AP	NS	AP	NS
ACh	*r*	**.475*****	**.341*****	**.325*****	**.339*****	-.024	.049	**.212****	.149	.085	.093	**.218****	.191	.154	**.283***
(nmol/ml)	*p*	**<.001**	**<.001**	**<.001**	**<.001**	.774	.663	**.009**	.149	.300	.374	**.007**	.064	.059	**.003**
	n	**151**	**94**	**151**	**94**	151	94	**151**	94	151	94	**151**	94	151	**94**
AChE	*r*	.044	-.023	.097	-.064	-.007	.038	.015	-.011	**.164***	**.246***	-.005	-.024	**.169***	.154
(Units)	*p*	.594	.842	.235	.535	.931	.712	.859	.912	**.044**	**.016**	.954	.817	**.038**	.137
	n	151	94	151	94	151	94	151	94	**151**	**94**	151	94	**151**	94
BChE	*r*	**-.213****	**-.308****	**-.247****	**-.221***	-.086	**-.271****	**-.170***	-.180	-.032	.004	**-.218****	-.197	.079	.042
(Units)	*p*	**.009**	**.008**	**.002**	**.032**	.296	**.032**	**.038**	.080	.695	.960	**.007**	.056	.337	.668
	n	**151**	**94**	**151**	**94**	151	**94**	**151**	94	151	94	**151**	94	151	94

*Correlation is significant at the 0.05 level, ** at the 0.01 level, *** at the 0.001 level (all 2-tailed and shown in bold text). ACh = Acetylcholine, AChE = Acetylcholinesterase, BChE = Butyrlcholinesterase, AP = All participants, NS = Never smokers only.

Figure 1

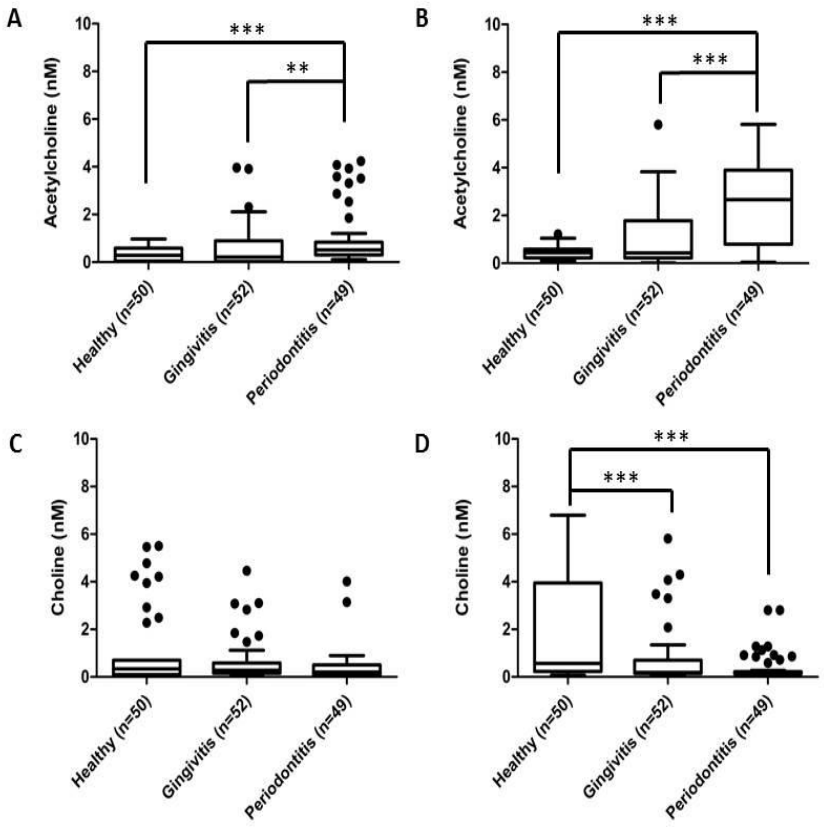

Figure 2