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### Periodontitis in twins : smoking, microbiological and immunological aspects

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## **Chapter 3**

### **Cigarette smoking enhances T cell activation and a Th2 immune response; an aspect of the pathophysiology in periodontal disease**

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## **Abstract**

**Background and Objective:** Smoking is a strong risk factor for periodontitis. Treated patients who smoke show increased risk for further periodontal breakdown, despite receiving maintenance care. Previous work indicated that such patients have a monocytic cytokine response favoring Th2 activity. The purpose of the present study was to investigate the T lymphocytic cytokine production representing Th1 and Th2 subpopulations in smokers and non-smokers.

**Material and Methods:** Venous blood was collected from 30 treated periodontitis patients (12 smokers) and 24 healthy subjects (12 smokers). Whole blood cell cultures were stimulated and interferon (IFN)- $\gamma$  and interleukin (IL)-13 were measured in the culture supernatants, representing type 1 and 2 Th subpopulations respectively.

**Results:** Unadjusted data showed that smokers had more lymphocytes, and higher levels of IFN- $\gamma$  and IL-13, irrespective of being periodontal patient. However in a multivariate analysis, increased IFN- $\gamma$  production was not significantly explained by smoking, while higher IL-13 was strongly explained by smoking (21%,  $p < 0.001$ ).

**Conclusion:** We suggest that the increased Th activity and specifically an elevated Th2 profile in smokers may constitute a risk for smoking patients which may induce conversion of periodontal stability into progressive disease. This phenomenon may be equally important in other conditions, where connective tissue and bone loss are hallmarks of disease pathophysiology.

## Introduction

Periodontitis is a chronic inflammatory disease of the supportive tissues of the teeth characterized by loss of periodontal attachment and alveolar bone. Approximately 10% of the population suffers from this condition and, if untreated, it may result in tooth loss. The inflammation is precipitated by the subgingival bacterial biofilm; a major pathogen associated with periodontitis is *Porphyromonas gingivalis* (*Pg*) (Slots & Ting 1999, van Winkelhoff et al. 2002). An important role in the onset and progression of periodontitis is played by the host immune response; susceptibility for periodontitis is greatly influenced by genetic and life style risk factors (Loos et al. 2005, Page et al. 1997). Cigarette smoking is considered as one of the most important life style risk factors for periodontitis (Bergstrom 2004); more clinical attachment loss and more alveolar bone loss have been observed in smoking than in non-smoking patients (Baharin et al. 2006, Grossi et al. 1995, Grossi et al. 1994, Xu et al. 2002). Moreover, smoking may be responsible for a less favorable outcome after periodontal treatment and is frequently associated with disease progression despite a strict periodontal maintenance care program (Baharin et al. 2006, Bergstrom 2004, Heasman et al. 2006). However exact mechanisms how smoking affects the host are still unclear.

In any inflammatory response, cytokines produced by monocytes and other antigen presenting cells (APC) drive polarization of non-committed T helper cells (Th) into either Th1 or Th2 (Kidd 2003, Mosmann & Coffman 1989). In particular, interleukin (IL)-12 produced by monocytes, macrophages, neutrophils and dendritic cells during innate immune responses, promotes naive T cells to differentiate into Th1, while the anti-inflammatory IL-10 cytokine favors Th2 differentiation (Kidd 2003). Th1 lymphocytes are characterized by the production of interferon (IFN)- $\gamma$  and IL-2, whereas Th2 cells produce mainly IL-13 and IL-4 (Kidd 2003). For the disease periodontitis it is generally accepted that the stable lesion (as in treated patients in the periodontal maintenance phase) is largely mediated by cells with a Th1 cytokine profile, while the progressive, unstable lesion involves Th2-like cells (Gemmell & Seymour 2004, Gemmell et al. 2007, Seymour et al. 1996). Th2 cells may induce expansion of activated B cells resulting in local antibody production and the pro-inflammatory IL-1, the latter inducing tissue destruction.

We hypothesize that smoking may convert stable periodontal lesions into unstable progressive lesions, among other causes, by tipping the Th1/Th2 balance towards a Th2 dominated host response. This may thus explain why smoking is a well known risk factor for further periodontal destruction despite supportive therapy (Baharin et al. 2006, Bergstrom 2004, Palmer et al. 2005). Some studies have investigated the effect of cigarette smoking and related toxic metabolites on the Th1/Th2 balance (Byron et al. 1994, Cheung et al. 1988, Cozen et al. 2004, Hagiwara et al. 2001, Ouyang et al. 2000, Simhan et al. 2005). From these studies, a shift towards a Th2 phenotype in smokers is most likely occurring. In our previous study we observed in smoking periodontal maintenance patients a type 2 monocytic response to lipopolysaccharide (LPS) compared to non-smokers (Torres de Heens et al. 2009). However, the effect of smoking on the actual Th1/Th2 balance has not been investigated.

Therefore the aim of the present study was to investigate whether T cells in smoking periodontal patients who are in maintenance therapy, show a more pronounced Th2 cytokine profile than similar non-smoker subjects. To confirm that the observed effect on T cells is indeed related to smoking and not mainly due to being a periodontal patient, we also recruited a periodontally healthy control group of smokers and non-smokers. We measured the representative Th1 and Th2 cytokines IFN- $\gamma$  and IL-13 after T cell stimulation with a combination of anti-CD3/anti-CD28.

## **Materials and Methods**

### **Subjects**

The study population consisted of patients and personnel from the Academic Center for Dentistry Amsterdam (ACTA). The selection criteria included: 1) Western European Caucasian descent, 2) age between 25 and 65 years and 3) being current smoker (smoking for at least 10 years with a consumption of  $\geq 10$  cigarettes/day [cig/day]) or being non-smoker (never smoker or ceased smoking more than 10 years before entering the study). Periodontal patients were subjects attending the periodontics clinic who had been treated for chronic periodontitis. These individuals were in a 3-monthly maintenance program. Periodontally healthy controls were recruited among subjects registered for restorative dental procedures or who visited the dental school for regular dental check-ups or who worked as personnel in the

dental school. For both patients and controls exclusion criteria were: 1) pregnancy, 2) presence of any other acute or chronic medical condition, including diabetes, viral, fungal or bacterial infections, or any systemic condition that may affect the periodontal status, 3) use of antibiotics within the last 6 months preceding the study, and 4) use of any medicine that may interfere with the lymphocyte function, such as non-steroidal anti-inflammatory drugs.

The Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam approved the study. Participants were informed both verbally and in writing about the purpose of the study, and provided signed informed consent.

### **Sample collection and whole blood cell cultures (WBCC)**

From each subject, venous blood was collected by venipuncture from the antecubital fossa in a sterile pyrogen-free blood collection tube containing sodium heparine (Vacuette, Greiner, Alphen a/d Rijn, The Netherlands). Heparinized venous blood was used and cultured at a final 1:10 dilution. Whole blood cell cultures (WBCC) were performed in a 96-well flat bottom microtitre plate (Nunc, Roskilde, Denmark). All cultures were carried out in endotoxin-free Iscove's modified Dulbecco's medium (IMDM, BioWhittaker, Verviers, Belgium), supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) (Gibco, Merelbeke, Belgium), 0.1% endotoxin-free fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands), and 15 U/ml sodium heparin (Leo Pharmaceutical Products B.V., Weesp, The Netherlands). To stimulate T lymphocytes a combination of a mouse monoclonal antibody against human CD3 (anti-CD3, CLB-T3/4.E, 1 µg/ml) and CD28 (anti-CD28 CLB-T3/4.E, 1 µg/ml) (both from Sanquin, Amsterdam, The Netherlands) was added to the aliquot of 200 µl of diluted blood, as previously described (Gerards et al. 2003). Cultures were performed in duplicate and unstimulated diluted whole blood served as a negative control. After 72 hours of incubation with anti-CD3/anti-CD28, supernatants were harvested and stored at -20 °C until cytokine analysis was performed.

Venous blood of all participants was also collected in an EDTA (K<sub>3</sub>)-containing tube (Becton Dickinson Vacutainer System, Meylan, France) for the determination of the total number of leukocytes, and leukocyte differentiation (neutrophils, lymphocytes, monocytes, eosinophils and basophils), with standard automated procedures (Cell-Dyn 4000, Hematology Analyzer, Abbott Laboratories,

Park, IL, U.S.A.) operated in the clinical chemistry laboratory of the neighboring hospital (Slotervaart, Amsterdam, The Netherlands).

### **Cytokine assays**

Supernatants of WBCC were analyzed for levels of IFN- $\gamma$  and IL-13 using commercially available ELISA kits (PeliKine Compact™ human ELISA kits, Sanquin) as described previously (van der Pouw-Kraan et al. 1993).

### **Statistical analysis**

Data analyses were performed with the SPSS 15.0 package (SPSS Inc., Chicago, IL, USA). Means, standard deviations and frequency distributions for all characteristics of the study population were calculated. Possible differences in these background parameters between 4 groups of subjects (smokers and non-smokers for both periodontal patients and controls) were analyzed with one-way ANOVA (post hoc testing with Bonferroni correction) or  $X^2$ -test, where appropriate. The outcome variables IFN- $\gamma$  and IL-13 in culture supernatants were compared between the 4 groups of subjects with non-parametric Kruskal-Wallis tests (post hoc testing with Bonferroni correction). For further explorative analysis, IFN- $\gamma$  and IL-13 values were first log-transformed since they were not normally distributed (Kolmogorov-Smirnov goodness-of-fit test  $p < 0.05$ ). A multivariate analysis (backward stepwise linear regression with  $p \leq 0.10$  to enter and  $p \leq 0.05$  to leave) was performed to identify factors explaining the observed variation in the IFN- $\gamma$  and IL-13 values. For these latter analyses predictor variables entered were smoking, periodontal patients, age, gender and number of lymphocytes.  $p$  values  $< 0.05$  were considered statistically significant.

## **Results**

Subject background characteristics are presented in Table 1. Cigarette smoking consumption reported by current smokers showed a mean of 14.8 cig/day during 33.9 years for those with periodontal disease and 17.6 cig/day on average during 20.6 years for controls. The levels of total peripheral leukocytes were significantly different among the 4 groups, with smokers having higher levels than non-smokers. Also the numbers of lymphocytes and monocytes were significantly different among the 4 groups, showing the same trends as total leukocytes (Table 1).

Table 1. Characteristics of the study population (smoking and non-smoking periodontal patients and controls).

	Patients		Controls	
	Smokers (N = 12 )	Non-smokers (N = 18)	Smokers (N = 12)	Non-smokers (N = 12)
Age (years)	53.6 ± 5.8	50.3 ± 3.9	40.4 ± 12.2	39.6 ± 11.1
Gender				
Female	7 (64%)	11 (61%)	7 (58%)	7 (58%)
Male	4 (36%)	7 (39%)	5 (42%)	5 (42%)
Smoking habits				
Number of cigarettes/day	14.8 ± 5.8	0	17.6 ± 5.8	0
Number of years smoking	33.9 ± 6.7	0	20.6 ± 11.6	0
Total Leukocytes (10 <sup>9</sup> /L) <sup>1</sup>	7.30 ± 0.84	6.12 ± 1.42	8.61 ± 2.10 <sup>2</sup>	6.55 ± 1.79
Neutrophils (10 <sup>9</sup> /L)	4.30 ± 0.76	3.48 ± 1.20	4.87 ± 1.87	3.91 ± 1.72
Lymphocytes (10 <sup>9</sup> /L) <sup>3</sup>	2.29 ± 0.32	1.96 ± 0.49	2.76 ± 0.54 <sup>4</sup>	1.90 ± 0.69
Monocytes (10 <sup>9</sup> /L) <sup>5</sup>	0.50 ± 0.17	0.47 ± 0.13	0.73 ± 0.26 <sup>6</sup>	0.54 ± 0.14
Eosinophils (10 <sup>9</sup> /L)	0.17 ± 0.11	0.14 ± 0.12	0.21 ± 0.13	0.17 ± 0.13
Basophils (10 <sup>9</sup> /L)	0.08 ± 0.02	0.02 ± 0.07	0.03 ± 0.04	0.01 ± 0.03

Values are means ± standard deviation or number (%) of subjects.

<sup>1</sup> overall ANOVA  $p=0.001$

<sup>2</sup> value is higher than for non-smoking patients ( $p=0.001$ ) and than for non-smoking controls ( $p=0.016$ )

<sup>3</sup> overall ANOVA  $p<0.001$

<sup>4</sup> value is higher than for non-smoking patients ( $p=0.001$ ) and than for non-smoking controls ( $p=0.001$ )

<sup>5</sup> overall ANOVA  $p=0.002$

<sup>6</sup> value is higher than for smoking patients ( $p=0.016$ ) and than for non-smoking patients ( $p=0.002$ )

WBCC were incubated with a combination of anti-CD3/anti-CD28 and supernatants were analyzed for IFN- $\gamma$  and IL-13 cytokine production, both indicators for the direction of the Th response (data presented in Fig 1). Overall a significant difference between the 4 groups was observed for IFN- $\gamma$  ( $p=0.012$ ). In Fig. 1A it can be seen that smokers in general had higher levels of IFN- $\gamma$  than non-smokers irrespective of periodontal disease. Post-hoc testing showed that smokers with periodontal disease produced higher levels than non-smoking controls. Similarly, among the 4 groups, there was an overall difference for IL-13 production in

supernatants of WBCC ( $p=0.001$ ); smokers produced more IL-13 irrespective of periodontal disease, and their levels were higher in post hoc testing than in non-smokers with periodontal disease.

Figure 1

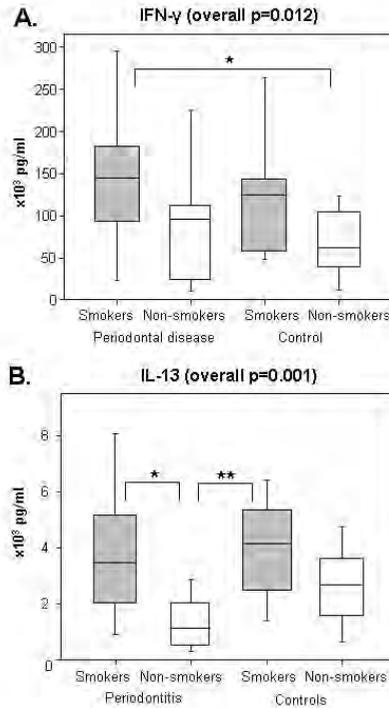


Figure 1. Boxplots for T cell cytokines IFN- $\gamma$  (panel A) and IL-13 (panel B), in supernatants from whole blood cell cultures from smoking and non-smoking subjects with and without periodontal disease after 72 hrs stimulation with a combination of anti-CD3/anti-CD28 mouse monoclonal antibody. Overall  $p$  values from non-parametric testing (Kruskal-Wallis) in heading; post hoc, adjusted for multiple testing: \*  $p<0.05$ , \*\*  $p<0.01$ .

To further explore which factors explained significantly the observed variation in IFN- $\gamma$  and IL-13 levels in the WBCC, we applied a linear regression analysis. For IFN- $\gamma$  a significant final model ( $p < 0.001$ ) was obtained, where all included explanatory variables were retained (Table 2). From this analysis we could infer that female gender and older age were associated with lower levels of IFN- $\gamma$ ; these variables explained together 25.4% of the variation. Also higher numbers of lymphocytes and being a periodontal patient correlated positively with IFN- $\gamma$  levels in the supernatants. Smoking contributed not significantly to higher levels of IFN- $\gamma$  ( $p = 0.080$ ); adjusted mean values of IFN- $\gamma$  for smokers and non-smokers were estimated to be 97.7 and  $67.8 \times 10^3$  pg/ml respectively (Table 3). IL-13 as independent variable in the regression model yielded the same results as above, since IL-13 was not retained ( $p = 0.236$ ), suggesting that the IFN- $\gamma$  lymphocyte subpopulation behaved independent of IL-13.

In the final regression model exploring the IL-13 levels which are indicative of a type 2 T cell reactivity (overall  $p < 0.001$ , Table 2), smoking was the strongest explanatory variable ( $p < 0.001$ ); the adjusted correlation coefficient for smoking with IL-13 was 0.457, explaining 20.9% of the variation. The adjusted mean values for smokers and non-smokers for IL-13 were estimated to be 3.49 and  $1.59 \times 10^3$  pg/ml respectively (Table 3). In addition to smoking, also periodontal patient was retained ( $p = 0.025$ , negatively correlated, explaining only 7.3%), while age, number of lymphocytes and gender were not of influence on the observed variation in the study population (Table 2). Interestingly, levels of IL-13 were not associated with IFN- $\gamma$ , as IFN- $\gamma$  levels were not included in the final model. This suggests that an IL-13 producing subpopulation of T lymphocytes behaves independently from the IFN- $\gamma$  subpopulation.

Table 2. Results of the multivariate analyses for IFN- $\gamma$  and IL-13 values in the supernatants of whole blood cell cultures, using IFN- $\gamma$  and IL-13 as dependent variable and age, gender, periodontal disease, number (no.) of lymphocytes and smoking status as predictor variables (N=54). In the model exploring IFN- $\gamma$ , IL-13 levels were also entered; in the model exploring IL-13, IFN- $\gamma$  levels were also entered.

	IFN- $\gamma$ <sup>1</sup>			IL-13 <sup>2</sup>		
	$\beta$ (95% CI)	Explained variance	<i>p</i> -value	$\beta$ (95% CI)	Explained variance	<i>p</i> -value
Smoking <sup>3</sup>	0.158 (-0.020, 0.336)	5.2%	0.080	0.457 (0.166, 0.518)	20.9%	<0.001
Periodontal patient <sup>4</sup>	0.295 (0.012, 0.396)	8.7%	0.038	-0.270 (-0.377, -0.026)	7.3%	0.025
Age	-0.315 (-0.190, -0.001)	9.9%	0.029	n.r.	-	-
No. of lymphocytes	0.297 (0.020, 0.316)	8.8%	0.027	n.r.	-	-
Gender <sup>5</sup>	-0.394 (-0.435, -0.114)	15.5%	0.001	n.r.	-	-

n.r. = not retained in final model

<sup>1</sup> final model: *p*<0.001, where IL-13 was not retained.

<sup>2</sup> final model: *p*<0.001, where IFN- $\gamma$  was not retained.

<sup>3</sup> in statistical analysis “non-smoker”=0, “smoker”=1.

<sup>4</sup> in statistical analysis “not a periodontal patient”=0, “being a periodontal patient”=1.

<sup>5</sup> in statistical analysis “female”=0, “male”=1.

Table 3. Adjusted mean values (95% confidence interval) for levels of IFN- $\gamma$  and IL-13 in supernatants from the ANCOVA model for all smokers and non-smokers, and *P<sub>adj</sub>*-values.

	Smokers (N = 24)	Non-smokers (N = 30)	<i>P<sub>adj</sub></i> -value
IFN- $\gamma$	97.7 (73.1 - 130.3)	67.8 (52.6 - 87.5)	0.080
IL-13	3.49 (2.58 - 4.72)	1.59 (1.21 - 2.08)	<0.001

Values are x 10<sup>3</sup> pg/ml

## Discussion

This study was conducted to investigate the influence of cigarette smoking on the type of Th cell response in periodontitis patients who are in maintenance phase. We added a control group of smokers and non-smokers to support that the observations in smokers are not due to the disease process itself. In a previous study we found that in the same type of smoking patients (receiving supportive periodontal care), T cell directing cytokines derived from monocytic cells showed a more pronounced type 2 profile than in non-smoking patients (Torres de Heens et al. 2009). In the present study we stimulated lymphocytes from smoking and non-smoking periodontal patients and similarly from control subjects to study whether indeed lymphocytes from smokers are more inclined to a type 2 phenotype. The overall results show more lymphocytes in blood of smokers and more lymphocytic cytokine production in smokers. We suggest that the increased IFN- $\gamma$  production in the present study is multifactorial, due to increased numbers of lymphocytes, periodontal status and strongly influenced by female gender and age, and not significantly by smoking. For IL-13 levels however, smoking was a strong explanatory variable and being a periodontal patient a minor explanatory variable, while it was not explained by a higher number of lymphocytes, age and gender. We suggest from these data that smoking stimulates the proliferation of a pronounced Th2-type cell subpopulation.

Important to note is that both IFN- $\gamma$  and IL-13 productions were independent of each other, indicative of two independent Th subpopulations. Moreover, the higher IL-13 levels are not due the disease process itself, as similar findings were in both the periodontal as well as the control study groups (Fig. 1); on the contrary, from the multivariate analysis on IL-13 levels, periodontal patient status is inversely correlated with IL-13. This latter observation and the results of the IFN- $\gamma$  multivariate analysis reflect that the controls and the treated periodontal patients showed a good periodontal health; IFN- $\gamma$  was positively associated with being a periodontal patient, suggestive for the previously described Th1-associated periodontal stability (Gemmell & Seymour 2004, Gemmell et al. 2007, Seymour et al. 1996).

Other studies have also investigated the effect of cigarette smoking and related toxic metabolites on the Th1/Th2 balance (Byron et al. 1994, Cheung et al. 1988, Cozen et al. 2004, Hagiwara et al. 2001, Ouyang et al. 2000, Simhan et al. 2005). For example, after *ex vivo* mitogen stimulation of peripheral blood mononuclear cells

(PBMC) from healthy individuals it appeared that smoking was associated with an increase in IL-13 and IL-4 production in supernatant of cell cultures (Byron et al. 1994, Cozen et al. 2004), whereas IFN- $\gamma$  did not differ between smokers and non-smokers (Cozen et al. 2004). Furthermore, smoking was associated with a lower number of Th1 cytokine-secreting cells in bronchoalveolar lavage fluid (BALF) in smokers compared to non-smokers (Hagiwara et al. 2001). Also, cigarette smoke extracts suppressed the production of IFN- $\gamma$  from isolated human PBMC and mouse spleen cells (Cheung et al. 1988, Ouyang et al. 2000). Thus, smoking seems an important factor in the Th1/Th2 immunoregulation, promoting a shift towards a Th2 phenotype (Byron et al. 1994, Cozen et al. 2004, Hagiwara et al. 2001). Notably, in a previous study we found in smoking periodontal maintenance patients a type 2 monocytic response to lipopolysaccharide (LPS) compared to non-smokers (Torres de Heens et al. 2009); in the current study in a newly recruited study population we observed that indeed smoking was a strong factor explaining increased levels of IL-13, the marker cytokine for Th2. It is thus highly likely that the action of cigarette smoking is related primarily to the activation of Th2 rather than Th1 lymphocytes, although smoking is associated with increased numbers of lymphocytes.

Previous observations in the literature have shown increased lymphocyte reactivity in smokers (Loos et al. 2004, Silverman et al. 1975). T cells play a fundamental role in periodontal disease (Gemmell & Seymour 2004, Gemmell et al. 2007). It has been suggested that susceptible individuals for periodontitis may have a predominant Th2 type response, with a stimulatory effect on B-cells, which in turn contribute to tissue breakdown, most likely through release of IL-1 from activated B-cells (Aoyagi et al. 1995, Bartova et al. 2000, Gemmell & Seymour 2004, Gemmell et al. 2007, Manhart et al. 1994). In addition, early publications have shown a bias towards Th2 cytokine production in periodontitis after challenge of immune cells with two major periodontal pathogens (Jotwani et al. 2003, Mahanonda et al. 2006). A Th1 type cell mediation in periodontal disease may reflect periodontal stability or health. With the current study we add new information that smoking is a factor promoting a Th2 type lymphocytic phenotype. This may make smoking periodontal patients in maintenance care vulnerable for renewed disease progression and may explain the frequently observed increase of relapse into active periodontitis (Baharin et al. 2006, Bergstrom 2004, Heasman et al. 2006).

This is the first report on the effect of smoking on the actual cytokine production by lymphocytes in periodontal patients and normal subjects. Our study provides evidence for earlier speculations that smoking appears to be associated with increased levels of Th2 cytokines (Byron et al. 1994, Cozen et al. 2004, Torres de Heens et al. 2009). The present results suggest that cigarette smoking can prime lymphocytes toward a more pronounced Th2 phenotype, which in turn may exacerbate or increase severity in periodontitis or may induce recurrence of disease in treated subjects, possibly resulting in a hyperactivity of B cells in the periodontal tissues. This phenomenon may be equally important in other conditions where connective tissue and bone loss are hallmarks of disease pathophysiology.

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