

# The Concentration of Interleukin 6 and Tumor Necrosis Factor Alpha in Saliva and Blood of Patients with Inactive Multiple Sclerosis And Coexisting Hashimoto's Thyroiditis

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# THE CONCENTRATION OF INTERLEUKIN 6 AND TUMOR NECROSIS FACTOR ALPHA IN SALIVA AND BLOOD OF PATIENTS WITH INACTIVE MULTIPLE SCLEROSIS AND COEXISTING HASHIMOTO'S THYROIDITIS

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**SUMMARY** – The concentration of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) in the blood is higher in patients with active multiple sclerosis (MS) compared to those with inactive disease. The concentration of IL-6 and TNF- $\alpha$  in the blood is higher in patients with Hashimoto's thyroiditis (HT) compared to those with a healthy thyroid. The aim of the study was to assess whether serum IL-6 and TNF- $\alpha$  levels correlated with saliva in patients with inactive MS and whether there was a difference in these groups of patients depending of thyroid status. We also examined the correlation of thyroid stimulating hormone (TSH) levels with thyroid status. The study included 54 patients in the inactive phase of MS. The level of cytokines in the blood was determined by chemiluminescence, and in saliva by ELISA. Blood and saliva IL-6 levels showed positive correlation, while blood and saliva TNF- $\alpha$  levels were not correlated. There was a significantly higher TSH level in patients with inactive MS with positive thyroid antibodies, without therapy, compared with those with negative antibodies.

**Key words:** *Interleukin-6; TNF- $\alpha$ , Multiple sclerosis; Hashimoto's thyroiditis; Saliva*

## Introduction

Elevated interleukin-6 (IL-6) levels in various autoimmune disorders suggest that IL-6 has a critical role in mediating disease initiation and/or progression<sup>1</sup>. IL-6 is a central player in physiological neuronal and glial function, as well as in neuroinflammatory pathways observed in central nervous system (CNS) diseases<sup>2</sup>.

Tumor necrosis factor-alpha (TNF- $\alpha$ ) is a pleiotropic cytokine increasingly recognized to regulate important physiological processes not only in the immune system but also in the brain<sup>3-5</sup>. Evidence for TNF- $\alpha$  involvement in multiple sclerosis (MS) includes identification of TNF- $\alpha$  in astrocytes, microglia, and endothelial cells, preferentially in acute and chronic active MS brain lesions, and in the cerebrospinal fluid (CSF) of MS subjects<sup>6-8</sup>. Hashimoto's thyroiditis (HT), the most common cause of hypothyroidism in developed countries, is an autoimmune disease in which thyroid cells are destroyed *via* cell and antibody-mediated immune processes<sup>9</sup>. A higher level of IL-6

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in patients with positive antithyroid antibodies has been observed independently of thyroid stimulating hormone (TSH) range.

Multiple sclerosis is an inflammatory autoimmune disease where myelin is damaged, and axonal degeneration, demyelination and inflammation occur in the CNS<sup>10</sup>. Elevated IL-6 levels have been reported in the CNS of MS patients and in a mouse experimental autoimmune encephalitis (EAE) model of MS<sup>11</sup>. Given that the literature reports elevated blood concentrations of IL-6 and TNF- $\alpha$  in patients with active MS, as well as in patients with HT, we compared IL-6 and TNF- $\alpha$  levels in patients with inactive MS and healthy thyroid with those in patients with inactive MS and HT. We compared these groups by determining blood and saliva concentrations. In addition to correlating saliva and blood, our goal was to investigate the impact of these two autoimmune diseases on IL-6 and TNF- $\alpha$  levels in blood and saliva. We also compared TSH level between the two groups of patients. Saliva is an important biological fluid the samples of which are easy to collect, noninvasive and inexpensive.

## Materials and Methods

This study was approved by the Ethics Committee of Sestre milosrdnice University Hospital Center (UHC) and Ethics Committee of the School of Dental Medicine, University of Zagreb, Zagreb, Croatia. All participants signed informed consent according to Helsinki II. We included 54 patients of both sexes, treated at Department of Neurology, Sestre milosrdnice UHC. All patients filled out a questionnaire with general data, age at diagnosis, duration of remission, therapy, supplements, diet, malignancy, smoking, and alcohol abuse. Patients with other autoimmune diseases and dental works were excluded. Inadequate specimens were also excluded. Of 54 patients, 38 had inactive MS and normal thyroid, and 16 had inactive MS and HT. In all patients, routine blood tests, TSH, free triiodothyronine (fT4), free thyroxine (fT3), thyroid peroxidase antibodies (A-TPO), thyroglobulin antibodies (A-TG), and C-reactive protein (CRP) were performed. Saliva and serum were collected for IL-6 and TNF- $\alpha$  determination. Whole saliva samples were collected with SalivaBio 2-mL cryovials and Saliva Collection Aid (exclusively from Salimetrics, State College, PA, USA), a collection device specifically designed to improve volume collection and increase participant compliance and validated

for use with saliva. Unstimulated, whole saliva is the gold standard in saliva collection. It was collected by passive drool technique to maintain consistency in the type of sample collected. Immediately after collection, samples were frozen at -20 °C. On the day of assay, the samples were thawed, vortexed and centrifuged for 15 minutes at 396 xg on a Rotina 35R centrifuge (Hettich, Kirchlengern, Germany). IL-6 and TNF- $\alpha$  in serum, as well as TNF- $\alpha$  in saliva were measured on an Immulite 1000 automatic immunochemistry analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany), with chemiluminescent immunoassay method. IL-6 in saliva was measured by a commercial kit (Salimetrics, PA, USA) using the enzyme-linked immunosorbent assay (ELISA). TNF- $\alpha$  and IL-6 levels were expressed in pg/mL.

Peripheral blood samples were collected by venipuncture in two Vacuette<sup>®</sup> red cap serum tubes with clot activator (Greiner Bio-One, Kremsmünster, Austria) for CRP, TSH, fT4, fT3, A-TPO, A-TG, IL-6, TNF- $\alpha$ , and one tube with K<sub>2</sub>EDTA, lavender cap for complete blood count (CBC).

Blood for serum testing was centrifuged for 10 minutes at 3500 g/min.

The levels of CRP, TSH, fT4, A-TPO, A-TG were analyzed immediately, and for IL-6 and TNF- $\alpha$ , two aliquots (2x550  $\mu$ m) of serum samples were immediately frozen at -20 °C until analysis. CRP was determined by immunoturbidimetry on an Architect c8000 clinical chemical analyzer (Abbott, IL, USA), using calibrators and controls. The recommended reference value for CRP is <5 mg/L. TSH, fT4 and fT3 were determined on an Abbott i2000 immunoassay analyzer (Abbott, IL, USA) by chemiluminescence method, and A-TPO and A-TG on Cobas e601 analyzer (Roche Diagnostics, Basel, Switzerland) by electrochemiluminescence method. CBC was determined on a DxH 520 (Beckman-Coulter, Brea, USA) analyzer. The recommended reference value of TSH is 0.35-4.94 mIU/L, fT4 9.01-19.05 pmol/L, A-TPO <34 kIU/L, and A-TG <115 kIU/L.

Serum IL-6 and TNF- $\alpha$  and salivary TNF- $\alpha$ , after being defrosted by leaving at room temperature until liquid state, were analyzed on Immulite1000 (Siemens Healthcare Diagnostics, Erlangen, Germany) by the chemiluminescent enzyme immunometric method using original Siemens reagents and adjustors according to the manufacturer's instructions.

Teeth were washed one hour before collecting specimen and 12-hour fasting, without any oral disease, injuries, and inflammation of oral cavity.

Statistical analysis was performed using IBM SPSS Statistics, version 22.

## Results

The study included 54 patients (43 women and 11 men). Serum and saliva were collected for IL-6 and TNF- $\alpha$  determination. Their mean age was  $37.56 \pm 13.41$  years, median 34.50 with normal distribution (Shapiro-Wilk, test of normality; sig=0.062;  $p > 0.05$ ). HT was found in 29.6% and normal thyroid in 70.4% of patients. There were no significant age and BMI differences between genders

(independent samples test). There was no significant difference between sexes according to HT incidence in MS patients ( $\chi^2$ -test; asymp. sig. 0.848). There was a statistically significant correlation between IL-6 levels measured in saliva and blood ( $r=0.465$ ;  $p < 0.01$ ), as well as between IL-6 measured in blood and TNF- $\alpha$  measured in blood ( $r=0.389$ ;  $p < 0.01$ ). Other variables showed no statistically significant correlation (Table 1). Analysis of IL-6 and TNF- $\alpha$  levels in saliva and serum in the group of patients with HT and the group of patients with normal thyroid yielded no significant correlation between the groups (independent samples test,  $p < 0.05$ ) (Table 2). Mean TSH level in all patient groups was 1.41 mg/mL. In the group of patients with normal thyroid and the group of patients with HT, the

Table 1. Correlation between IL-6 measured in saliva and blood, and between IL-6 in blood and TNF- $\alpha$  in blood ( $N=48$ )

		IL-6 saliva	IL-6 blood	TNF- $\alpha$ saliva	TNF- $\alpha$ blood
IL-6 saliva	Pearson r	1	0.465**	0.119	0.022
	Sig.		0.001	0.420	0.880
IL-6 blood	Pearson r	0.465	1	-0.074	0.389
	Sig.	0.001		0.619	0.006
TNF- $\alpha$ saliva	Pearson r	0.119	-0.074	1	0.031
	Sig.	0.420	0.619		0.836
TNF- $\alpha$ blood	Pearson r	0.022	0.389	0.031	1
	Sig.	0.880	0.006	0.836	

\*\*Correlation significant at the 0.01 level (2-tailed); IL-6 = interleukin 6; TNF- $\alpha$  = tumor necrosis factor alpha; Pearson r = Pearson correlation coefficient; Sig. = statistical significance

Table 2. Independent samples test: no significant correlation between IL-6 and TNF- $\alpha$  levels in blood and saliva in normal thyroid and HT groups

	F	Sig.	t	df	Sig.	Mean difference	SE of difference	95% CI of difference lower	95% CI of difference upper
IL-6 saliva	2.567	0.115	0.772 0.689	50 20.940	0.444 0.499	2.30546 2.30546	2.98535 3.34773	-3.3691 -4.658	8.302 9.269
IL-6 blood	2.002	0.163	1.196 1.086	50 21.602	0.237 0.289	0.7360 0.7360	0.6154 0.6776	-0.500 -0.671	1.972 2.142
TNF- $\alpha$ saliva	0.627	0.432	-0.477 -0.456	50 23.664	0.635 0.653	-1.9757 -1.9757	4.1401 4.3366	-10.291 -10.933	6.340 6.981
TNF- $\alpha$ blood	0.935	0.338	0.470 0.415	50 20.587	0.640 0.683	0.223 0.223	0.475 0.539	-0.731 -0.898	1.178 1.345

IL-6 = interleukin 6; TNF- $\alpha$  = tumor necrosis factor alpha; HT = Hashimoto's thyroiditis; F = test statistic of Leven's test; t = computed test statistic; df = degrees of freedom; Sig. = statistical significance; SE = standard error; 95% CI = 95% confidence interval

Table 3. Independent sample test: TSH level in the group of HT patients and group with normal thyroid

	F	Sig.	t	df	Sig.	Mean difference	SE of difference	95% CI of difference lower	95% CI of difference upper
TSH	5.149	0.027	2.396	52	0.020	0.703	0.294	0.114	1.293
			2.159	22.95	0.042	0.703	0.326	0.0293	1.378

TSH = thyroid stimulating hormone; HT = Hashimoto's thyroiditis; F = test statistic of Leven's test; t = computed test statistic; df = degrees of freedom; Sig. = statistical significance; SE = standard error; 95% CI = 95% confidence interval

Table 4. Correlation between salivary CRP and IL-6 levels, and between blood CRP and IL-6 levels (N=48)

	IL-6 saliva	IL-6 blood	TNF- $\alpha$ saliva	TNF- $\alpha$ blood	CRP
IL-6 saliva	1	0.465** 0.001	0.119 0.420	0.022 0.880	0.339* 0.018
IL-6 blood	0.465** 0.001	1	-0.074 0.619	0.389** 0.006	0.372** 0.009
TNF- $\alpha$ saliva	0.119 0.420	-0.074 0.619	1	0.031 0.836	-0.077 0.601
TNF- $\alpha$ blood	0.022 0.880	0.389** 0.006	0.031 0.836	1	0.281 0.053
CRP	0.339* 0.018	0.372** 0.009	-0.077 0.601	0.281 0.053	1

CRP = C-reactive protein; IL-6 = interleukin 6; TNF- $\alpha$  = tumor necrosis factor alpha; \*\*correlation significant at the 0.01 level (2-tailed); \*correlation significant at the 0.05 level (2-tailed)

Table 5. Correlation between EDSS, IL-6 and TNF- $\alpha$  (N=48)

	IL-6 saliva	IL-6 blood	TNF- $\alpha$ saliva	TNF- $\alpha$ blood	EDSS
IL-6 saliva	1	0.465** 0.001	0.119 0.420	0.022 0.880	-0.104 0.481
IL-6 blood	0.465** 0.001	1	-0.074 0.619	0.389** 0.006	-0.036 0.810
TNF- $\alpha$ saliva	0.119 0.420	-0.074 0.619	1	0.031 0.836	-0.222 0.130
TNF- $\alpha$ blood	0.022 0.880	0.389** 0.006	0.031 0.836	1	0.175 0.234
EDSS	-0.104 0.481	-0.036 0.810	-0.222 0.130	0.175 0.234	1

EDSS = Expanded Disability Status Scale; IL-6 = interleukin 6; TNF- $\alpha$  = tumor necrosis factor alpha; \*\*correlation significant at the 0.01 level (2-tailed)

mean TSH level was 1.199 (SD 0.902) and 1.903 (SD 1.164), respectively. There was a significant difference in TSH level between the group of patients with HT and the group with normal thyroid (independent samples test, sig. 0.042) (Table 3). There was no

correlation between TSH range and IL-6 and TNF- $\alpha$  level in blood and saliva. No significant difference was recorded between CRP level and thyroid status (independent samples test) (Table 4). There was a significant correlation between CRP level and IL-6

level in saliva ( $p < 0.05$ ), and significant correlation between CRP level and IL-6 level in serum ( $p < 0.01$ ) (Table 4). There was no significant difference in the Expanded Disability Status Scale (EDSS) between patients with normal thyroid (independent samples test;  $r = 0.552$ ) and those with HT (independent samples test;  $r = 0.581$ ). There was no statistically significant correlation between EDSS and IL-6 and TNF- $\alpha$  in any patient group (Table 5). There were 37% of smokers and 63% of no smokers. Smokers and no smokers showed no correlations between saliva and serum levels of IL-6 and TNF- $\alpha$  in the group with normal thyroid and the group with HT.

## Discussion

Serum levels of IL-6 and TNF- $\alpha$  are elevated in the active phase of MS. Several studies have shown that B cells of patients with MS have defects in the balance of their cytokine expression, with a propensity for overproduction of inflammatory cytokines (TNF- $\alpha$  and IL-6) and deficit in the production of anti-inflammatory cytokines<sup>12</sup>.

Tumor necrosis factor alpha has been implicated in the pathogenesis of several human CNS disorders including MS<sup>13</sup>. Elevated IL-6 levels have been reported in the CNS of MS patients<sup>11</sup>. In our study, we included 54 patients with inactive MS. Since our target group were patients with inactive MS, we expected that the mere existence of MS in a patient would not affect cytokine levels<sup>14</sup>, which we confirmed since median IL-6 in serum was 0.35, mean 5.99; median IL-6 in saliva was 1.5 and mean 2.21; mean serum TNF- $\alpha$  was 6.31, median 6.00, indicating that most of our patients had IL-6 levels in both saliva and blood within the reference interval. Mean saliva TNF- $\alpha$  was 25.406 and median 21.75, analyzed by the chemiluminescent enzyme immunometric method, as expected. Because some studies have shown that serum levels of IL-6 and TNF- $\alpha$  are higher in patients with elevated thyroid antibody levels<sup>15</sup>, we expected the levels of IL-6 and TNF- $\alpha$  to be higher in patients with inactive MS and HT than in patients with inactive MS and a healthy thyroid. HT was found in 29.6% and normal thyroid in 70.4% of patients. In our research, analysis of IL-6 and TNF- $\alpha$  levels in serum and saliva of patients with HT and in the group of patients with normal thyroid showed no significant correlation between the groups. The incidence of HT in women is 4-10 times higher than in men<sup>16</sup>. In our study, there was no significant

difference between sexes regarding HT incidence in MS patients. There was a statistically significant correlation between IL-6 measured in saliva and in serum in our study, as well as in the study by Hanneman *et al.*, where the association between IL-6 in saliva and plasma was moderate but significant<sup>17</sup>. A statistically significant correlation was also found between serum IL-6 and serum TNF- $\alpha$ . There was no significant correlation between salivary and blood level of TNF- $\alpha$ <sup>17</sup>. Analysis of IL-6 and TNF- $\alpha$  levels in serum and saliva in the group of patients with HT and the group of patients with normal thyroid yielded no significant correlation between the groups. This could be explained by the fact that all patients had inactive phase of MS and HT without symptoms and therapy, only with positive thyroid antibodies. There was a significant difference in TSH level between the group of patients with HT and the group with normal thyroid. The most common laboratory findings demonstrated elevated TSH and low T4 levels, coupled with an increase in anti-TPO antibodies<sup>9</sup>.

There was no correlation between TSH range and IL-6 and TNF- $\alpha$  in blood and saliva in either group, which is opposite to some findings reported by Marchiori *et al.*<sup>18</sup>.

A significant correlation between CRP level and level of IL-6 in serum and saliva was found in our study, as well as in the study by Rasic *et al.*, where IL-6 and CRP serum levels showed very similar trends<sup>19</sup>. EDSS showed no correlation with thyroid status and the levels of IL-6 and TNF- $\alpha$  in saliva and blood.

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### Sažetak

## KONCENTRACIJA INTERLEUKINA 6 I FAKTORA TUMORSKE NEKROZE ALFA U SLINI I KRVI BOLESNIKA S NEAKTIVNOM MULTIPLOM SKLEROZOM I SUPOSTOJEĆIM HASHIMOTOVIM TIREOIDITISOM

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Koncentracija interleukina 6 (IL-6) i faktora tumorske nekroze alfa (TNF- $\alpha$ ) u krvi je veća u bolesnika s aktivnom multiplom sklerozom (MS) u odnosu na one s neaktivnom bolešću. Koncentracija IL-6 i TNF- $\alpha$  u krvi je veća kod bolesnika s Hashimotovim tireoiditisom (HT) u odnosu na one sa zdravom štitnjačom. Cilj istraživanja bio je procijeniti koreliraju li serumske razine IL-6 i TNF- $\alpha$  s onima u slini kod bolesnika s neaktivnom MS i postoji li razlika među navedenim skupinama bolesnika ovisno o statusu štitnjače. Također smo ispitali korelaciju razine tireoidnog stimulirajućeg hormona (TSH) sa statusom štitnjače kod svih bolesnika. U istraživanje su uključena 54 bolesnika u neaktivnoj fazi MS. Razina citokina u krvi je određena metodom kemiluminiscencije, a u slini metodom ELISA. Pozitivno je korelirala razina IL-6 u krvi i slini, dok razina TNF- $\alpha$  u krvi i slini nije korelirala. Značajno je veća bila razina TSH u bolesnika s neaktivnom MS s pozitivnim protutjelima na štitnjaču, bez terapije, u odnosu na one s negativnim protutjelima.

Ključne riječi: *Interleukin-6; TNF- $\alpha$ ; Multipla skleroza; Hashimotov tireoiditis; Slina*