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# ХЕМОКИНЫ CCL17 И CCL22 ПРИ САРКОИДОЗЕ

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**Резюме.** В иммунопатогенезе саркоидоза и механизмах образования гранулем принимают участие различные клетки иммунной системы и продуцируемые ими цитокины. В настоящее время активно изучается роль хемокинов, идет поиск ключевых молекул, важных для привлечения клеток иммунной системы в очаги поражения и формирования гранулем, а также влияющих на исходы процесса гранулемообразования. Целью исследования явилось определение уровней CCL17/TARC и CCL22/MDC хемокинов в плазме крови больных саркоидозом, не получавших иммуносупрессивную терапию. Что является актуальной задачей для уточнения ряда аспектов иммунопатогенеза заболевания, а также для поиска информативных клинико-лабораторных критериев оценки активности и прогноза заболевания. Были исследованы образцы плазмы крови больных саркоидозом (n = 52). У 37% (19/52) отмечалось острое, а у 63% (33/52) – хроническое течение заболевания. Контролем служили образцы периферической крови, полученные от 22 практически здоровых добровольцев. Концентрации хемокинов (пг/мл) определялись методом мультиплексного анализа по технологии xMAP (Luminex), тест-системы Milliplex MAP (Millipore, США). У обследованных больных обнаружено достоверно повышенное содержание хемокинов относительно здоровых лиц: CCL17 – 78,24 пг/мл против 26,24 пг/мл, $p < 0,001$; CCL22 – 660,60 пг/мл против 405,00 пг/мл, $p < 0,001$. Анализ клинико-лабораторной значимости уровней хемокинов в плазме крови обследованных больных саркоидозом выявил параметры их чувствительности и специфичности. У больных с острым течением саркоидоза они составили: для CCL17 – 63% и 78%, CCL22 – 63% и 91%; при хроническом: CCL17 – 58% и 83%, CCL22 – 67% и 86% соответственно. У больных с хроническим течением заболевания установлена прямая положительная связь между уровнем ангиотензин-превращающего фермента (АПФ) и концентрацией хемокинов: для CCL17 ($r = 0,530$; $p = 0,003$), для CCL22 ($r = 0,446$; $p = 0,014$). У больных саркоидозом с признаками системности заболевания был достоверно повышен уровень CCL17: 102,82 пг/мл против 32,72 пг/мл, $p = 0,011$. Уровни хемокина CCL17 были достоверно повышены у больных с признаками гепатомегалии по сравнению с больными, не имеющими изменений данного органа: 130,73 пг/мл против и 51,60 пг/мл, $p = 0,022$. При наличии спленомегалии относительно больных без таких признаков отмечалось повышение концентраций хемокинов: CCL17 – 249,18 пг/мл против 46,87 пг/мл,

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p = 0,002; CCL22 – 1271,40 пг/мл против 660,63 пг/мл, p = 0,003. Таким образом, уровни хемокинов CCL17 и CCL22 при саркоидозе могут быть использованы в качестве дополнительных прогностических маркеров при хроническом течении саркоидоза с разной степенью активности клинических особенностей, в том числе, с наличием или отсутствием системных проявлений заболевания.

*Ключевые слова: саркоидоз, хемокины, CCL17, CCL22, плазма крови*

# CHEMOKINES CCL17 AND CCL22 IN SARCOIDOSIS

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**Abstract.** Various immune cells as well as related cytokines are involved in immunopathogenesis of sarcoidosis and mechanisms of granuloma development. Currently, a role for chemokines in sarcoidosis has been extensively investigated, which is paralleled with a search for key molecules necessary for recruiting immune cells to intrusion site and granuloma formation as well as affecting outcome of the latter. Our study was aimed for determining level of plasma CCL17/TARC and CCL22/MDC chemokines in patients with sarcoidosis who received no immunosuppressive therapy is of high priority for clarifying some aspects in underlying immunopathogenesis as well as seeking out for secure clinical and laboratory criteria for assessing activity and disease prognosis. We studied peripheral blood plasma samples of the patients with sarcoidosis (n = 52). In 37% (19/52), they exhibited acute clinical manifestations, and 63% (33/52) had chronic sarcoidosis. The control group included peripheral blood samples from healthy volunteers (n = 22). The chemokine concentrations (pg/ml) were determined by multiplex analysis using xMAP technology (Luminex), and Milliplex MAP test system (Millipore, USA). In the patients with sarcoidosis, significantly higher levels of chemokines were shown relative to healthy volunteers: CCL17 – 78.24 pg/ml *vs* 26.24 pg/ml, p < 0.001; CCL22 – 660.60 pg/ml *vs* 405.00 pg/ml, p < 0.001. Evaluation of clinical and laboratory diagnostic characteristics for plasma chemokine levels in sarcoidosis patients allowed to assess their sensitivity and specificity. The respective values were as follows: in acute sarcoidosis: for CCL17 – 63% and 78%, CCL22 – 63% and 91%; in chronic sarcoidosis: CCL17 – 58% and 83%, CCL22 – 67% and 86%, respectively. In chronic sarcoidosis the levels of this chemokine correlated with the activity of angiotensin-converting enzyme (ACE), for CCL17 (r = 0.530; p = 0.003), for CCL22 (r = 0.446; p = 0.014). Patients with systemic lesions *vs* no systemic lesions (sarcoidosis of the respiratory system only) had significantly elevated CCL17 level: 102.82 pg/ml *vs* 32.72 pg/ml, p = 0.011. The concentration of chemokine CCL17 was significantly increased in patients with *vs* without signs of hepatomegaly: 130.73 pg/ml *vs* 51.60 pg/ml, p = 0.022. Levels of chemokines was significantly increased in patients with *vs* without ultrasound signs of splenomegaly comprising: for CCL17 – 249.18 pg/ml *vs* 46.87 pg/ml, p = 0.002; for CCL22 – 1271.40 pg/ml *vs* 660.63 pg/ml, p = 0.003. Thus, it should be noted that the peripheral blood plasma level of chemokines CCL17 and CCL22 may be used as additional prognostic markers in chronic sarcoidosis with varying scoring of clinical signs including with/without systemic disease manifestations.

*Keywords: sarcoidosis; chemokines; CCL17; CCL22; peripheral blood plasma*

## Introduction

Sarcoidosis is a multisystem disease of unknown etiology, featured with heterogenous clinical manifestations and outcomes, morphologically belonging to granulomatous diseases and characterized by developing specific necrosis-free sarcoid granuloma in diverse organs, preferentially in the lungs and bronchopulmonary lymph nodes [10, 18, 23]. Various immune cells as well as related cytokines and chemokines are involved in immunopathogenesis of sarcoidosis and mechanisms of granuloma development. Sarcoid granulomas contain macrophages able to fuse and form multinucleated giant cells as well as

helper $CD4^+$T-cells (Th), whereas cytotoxic $CD8^+$T-cells (Tcyt), regulatory T-cells (Tregs), fibroblasts and B-cells reside on periphery [23]. Still, it is unclear what cell types largely play a crucial pathogenetic role in sarcoidosis. In particular, granuloma formation and macrophage activation in site of inflammation were shown to be linked to type 1 Th-cells (Th1) as well as plastic subsets of type 17 Th-cells (Th17) and relevant cytokines [6, 9, 26].

A directed cell migration to the site of intrusion is mediated by surface expression of specific chemokine receptors able to ligate tissue-derived chemotactic cytokines (chemokines). Currently, a role for chemokines in sarcoidosis has been extensively investigated, which is paralleled with a search for key molecules necessary for recruiting immune cells to intrusion site and granuloma formation as well as affecting outcome of the latter [1, 2, 3, 14, 16, 17, 18, 19]. Such molecules, in turn, are related to resolution of granuloma development and disease remission, or, inversely, to triggering fibrogenesis in affected organs [12, 16, 18, 21].

Chemokines CCL17/TARC (thymus and activation-regulated chemokine) and CCL22/MDC (macrophage-derived chemokine) recruit Th-cells bearing surface chemokine receptor CCR4 from the circulation to inflammatory foci [11, 18, 24]. CCR4 receptor is expressed on Th2-cells, Tregs as well as skin-migrating T-cells. Upon formation of inflammatory focus in the connective tissue beneath skin layers, CCL17 is expressed by dermal microvascular endothelial cells, whereas CCL22 is mainly found in dermal dendritic cells. In this regard, key function for CCL17 is recruitment and penetration of lymphocytes through vascular wall in the immediate vicinity to inflammatory site, whereas CCL22 mainly accounts for directed cell migration within the connective tissue outside blood vessels [8].

The level of CCL17 chemokine is an important marker for assessing ongoing atopic dermatitis, bullous pemphigoid and other skin disorders [22]. Severe progressive sarcoidosis was associated with parallel rise in CCL17 level and peripheral blood $CCR4^+CD4^+$T-cells compared to apparently healthy subjects, as well as in biopsy histology samples from damaged skin containing sarcoid granulomas [18, 20]. Dynamic three-year monitoring of sarcoidosis patients evidences about prognostic importance of elevated CCL17 level related to more aggravated clinical course, lung injury, and transition to fibrogenesis stage in affected tissues [18].

Chemokines CCL17 and CCL22 contribute to skewing towards M2-polarized macrophage phenotype exerting pro-fibrotic properties and expressing a whole set of cytokines and chemokines, including CCL17 and CCL22 [18, 21]. The latter chemokines were examined in activating fibrogenesis both in experimental models and clinical practice in patients with sarcoidosis, idiopathic pulmonary fibrosis and some other diseases [7, 12, 13, 18, 21].

Thus, assessing level of peripheral blood plasma CCL17 and CCL22 chemokines in patients with sarcoidosis who received no immunosuppressive therapy is of high priority for clarifying some aspects in underlying immunopathogenesis as well as seeking out for secure clinical and laboratory criteria for assessing activity and disease prognosis.

## Materials and methods

Patients with sarcoidosis (n = 52) aged 20-67 years, who received no immunosuppressive therapy including systemic steroid therapy and plasmapheresis were enrolled to the study. Blood samples were collected after peripheral vein puncture into vacuum test tube added with $K_3$EDTA.

Sarcoidosis was verified based on histology examination in 73% (38/52) patients and clinical and chest X-ray data in 27% (14/52) subjects. All patients were subdivided into two groups: acute onset sarcoidosis (Löfgren's syndrome, n = 19) and chronic onset sarcoidosis (non-Löfgren's syndrome, n = 33). In the latter case, diagnosis was verified by histology examination in 94% cases (31/33). All sarcoidosis patients were examined in the Department of the Research Institute of Interstitial and Orphan Lung Diseases at the First St. Petersburg State I. Pavlov Medical University of the Ministry of Healthcare of Russian Federation. In control group, samples of peripheral blood were collected from 22 age- and sex-matched apparently healthy subjects. All subjects provided informed consent, studies were conducted in accordance with the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects with 2013 Amendments, Clinical Practice Rules in the Russian Federation approved by the Order of the Ministry of Healthcare of the Russian Federation dated of 19.06.2003, No. 266, and Good Clinical Practice Rules in the Russian Federation approved by the Order of the Ministry of Healthcare of the Russian Federation dated of 01.04.2016, No. 200n.

Peripheral blood plasma cytokine level was analyzed in parallel with assessing clinical, instrumental and laboratory parameters. While examining patients, there were assessed complaints, symptoms, systemic organ injuries, elevated pulmonary artery pressure (pPA, mm Hg) according to echocardiographic examination (Echo-CG), enlarged lymph nodes, aggravated pulmonary changes, spread of injury foci, emergence and progression of fibrotic signs and other manifestations of unfavorable sarcoidosis course according to multispiral computed tomography (MSCT), as well as change in pulmonary function test by evaluating pulmonary volume. Intensity of

sarcoidosis was assessed by measuring activity of angiotensin converting enzyme (ACE, = 1IU/ml) in colorimetric assay with peptide substrate. Positive data were considered at ACE activity > 70 IU, reference level for subjects > 18 years of age was within the range 20-70 ACE IU. Detailed description and subsequent group subdivision based on ACE activity were published earlier [15].

Concentration of plasma chemokines CCL17/TARC and CCL22/MDC (pg/ml) was measured by using xMAP® Technology-based multiplex assay (Luminex, USA), commercially available kit Milliplex MAP (Millipore, USA) with magnetic micro-beads Milliplex Mag (USA), according to the manufacturer's instruction. Data were recorded and analyzed on Luminex MAGPIX Instrument System (Luminex, USA).

Statistical processing was carried out by using software suites Statistica 8.0 (StatSoft, USA) and GraphPad Prism 5.00 for Windows (GraphPad Prism Software Inc., USA). The data on relative and absolute count of various T-cell subsets were presented as median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). A nonparametric Mann–Whitney test was used for comparing data samples, whereas correlation analysis was performed using the r-Spearman rank correlation coefficient. Informativity of parameters, comparison of two distinct parameters and selection of optimal partition point were assessed by analyzing ROC (receiver-operating-characteristic)-curve followed by calculating area under ROC-curve (AUC). In addition, parameters of diagnostic sensitivity and specificity were described.

## Results and discussion

It was found that among patients with sarcoidosis (n = 52) acute and chronic onset disease was observed in 37% (19/52) and 63% (33/52) cases, respectively. All patients with sarcoidosis *vs* healthy volunteers had significantly elevated level both for CCL17 and CCL22 chemokines.

The level of CCL17 comprised 78.24 (35.72-167.40) pg/ml compared to volunteers showing 26.24 (13.28-49.55) pg/ml ($p < 0.001$), whereas CCL22 reached up to 660.60 (417.40-914.60) pg/ml *vs* 405.00 (287.50-508.00) pg/ml ($p < 0.001$).

While comparing chemokine level in patients with acute (group 1) and chronic (group 2) onset with control group it was found as follows: CCL17 was significantly elevated compared to control group comprising up to 58.51 (31.80-169.10) and 60.02 (35.99-124.70) *vs* 26.24 (13.28-49.55) pg/ml ($p = 0.005$ and $p = 0.001$), respectively. In contrast, no inter-group difference was found for CCL17 level ($p = 0.894$). On the other hand, level of CCL22 was also increased in group 1 and group 2 *vs* control group reaching up to 621.30 (348.50-840.80) and 660.60 (438.20-1073.00) *vs* 405.00 (287.50-508.00) pg/ml ($p = 0.007$ and $p < 0.001$). Finally, no difference in CCL22 level between patients with acute *vs* chronic onset sarcoidosis was observed ($p = 0.582$).

The informativity of the examined laboratory parameters was assessed using ROC-analysis in both acute and chronic onset sarcoidosis:

– acute onset sarcoidosis: sensitivity for CCL17 was 63%; specificity – 78%; AUC = 0.771; criterion > 49,01 pg/ml; $p = 0.005$; sensitivity for CCL22 – 63%; specificity – 91%; AUC = 0.750; criterion > 536.30 pg/ml; $p = 0.006$.

– chronic onset sarcoidosis: sensitivity for CCL17 – 58%; specificity – 83%; AUC = 0.783; criterion > 51.43 pg/ml; $p < 0.001$. Sensitivity for CCL22 – 67%; specificity – 86%; AUC = 0.780; criterion > 520.90 pg/ml; $p < 0.001$.

It is worth noting that in chronic onset sarcoidosis with relatively low-grade activity characterized with normal ACE magnitude (< 70 ACE IU), the level of chemokines showed no significant difference from those found in volunteers that for CCL17 reached 29.03 (25.25-51.60) pg/ml (volunteers – 26.24 (13.28-49.55); for CCL22 – 499.59 (312.87-639.92) pg/ml (volunteers – 405.00 (287.50-508.00).

In contrast, sarcoidosis patients with elevated ACE level (≥ 70 ACE IU) had these chemokines at significantly higher level compared to those with normal ACE concentration, and reached for CCL17 – 87.00 (37.86-118.77) *vs* 29.03 (25.25-51.60) pg/ml, $p = 0.039$; for CCL22 – 734.48 (505.82-1063.44) *vs* 499.59 (312.87-639.92) pg/ml, $p = 0.044$.

Thus, concentration of chemokines CCL17 and CCL22 in the peripheral blood of patients with sarcoidosis was significantly increased during active inflammatory process that was confirmed by correlation analysis in patients with chronic disease course: a direct positive relation found between the ACE level and chemokine concentration: for CCL17 the value Spearman's correlation coefficient was $r = 0.530$; $p = 0.003$, for CCL22 – $r = 0.446$; $p = 0.014$.

To assess a potential role of such chemokines in formation of systemic lesions during sarcoidosis, the group of patients with chronic onset sarcoidosis was divided into two subgroups: subgroup 1 – no systemic lesions (sarcoidosis of the respiratory system only), subgroup 2 – with systemic lesions.

Patients from subgroup 2 (systemic lesions) *vs* subgroup 1 had significantly elevated CCL17 level: 102.82 (51.60-162.42) pg/ml *vs* 32.72 (17.75-60.02) pg/ml, $p = 0.011$.

There were identified groups of patients with/without signs of hepatosplenomegaly to conduct higher precision analysis of systemic manifestations in sarcoidosis by comparing chemokine levels.

**TABLE 1. PERIPHERAL BLOOD PLASMA LEVELS OF CHEMOKINES CCL17/TARC AND CCL22/MDC (pg/ml) IN PATIENTS WITH SARCOIDOSIS (n = 32) WITH/WITHOUT HEPATO-SPLENOMEGALY, Me ($Q_{0,25}$-$Q_{0,75}$)**

| Chemokine | Signs of hepatomegaly | | Statistically significant (p) | Signs of splenomegaly | | Statistically significant (p) |
|---|---|---|---|---|---|---|
| | Yes (n = 9) | No (n = 23) | | Yes (n = 9) | No (n = 23) | |
| **CCL17/TARC** | 130.73 (102.82-249.18) | 51.60 (27.58-92.35) | p = 0.022 | 249.18 (102.82-379.39) | 46.87 (27.58-92.35) | p = 0.002 |
| **CCL22/MDC** | 1063.44 (734.48-1414.98) | 660.63 (398.83-840.84) | p = 0.068 | 1271.40 (906.14-1417.06) | 660.63 (347.64-835.85) | p = 0.003 |

It was noted that such chemokines were significantly elevated in patients with *vs* without hepatosplenomegaly (Table 1).

The concentration of chemokine CCL17 was significantly increased in patients with *vs* without signs of hepatomegaly: 130.73 pg/ml (102.82-249.18) and 51.60 pg/ml (27.58-92.35), p = 0.022. Along with that, level of CCL17 was significantly increased in patients with *vs* without ultrasound signs of splenomegaly comprising 249.18 pg/ml (102.82-379.39) *vs* 46.87 pg/ml (27.58-92.35) respectively, (p = 0.002). Moreover, similar pattern was also observed for CCL22: 1271.40 pg/ml (906.14-1417.06) *vs* 660.63 pg/ml (347.64-835.85) (p = 0.003).

The ROC analysis allowed to establish the peak value of the informativity for plasma CCL17 level in patients with sarcoidosis with and without splenomegaly, with sensitivity – 89%; specificity – 65%; AUC = 0.865; criterion > 78.24 pg/ml; p = 0.002.

Patients with chronic onset sarcoidosis were found to have significantly increased concentration of CCL17 and CCL22 chemokines coupled to splenomegaly signs: CCL17 was 176.57 (90.57-373.32) pg/ml and 49.23 (27.58-99.62) pg/ml, p = 0.009; CCL22: 1221.06 (714.88-1416.025) pg/ml and 650.28 (385.59-790.76) pg/ml, respectively, p = 0.011.

Previously, it was published that high amount of serum chemokine CCL17 was observed in some disorders characterized by Th2-dependent mechanisms of immune response. Among those are atopic dermatitis, eosinophilic pneumonia, bronchial asthma etc. [18, 22, 25, 27]. The data obtained over the last years provided new insights into M2 macrophage polarization triggering tissue remodeling for cytokines from Th2 T-cells expressing chemokine receptor CCR4 [13]. Such chemokines examined by us are involved in activation and recruitment of regulatory $CCR4^+$T-cells profoundly contributing to immune regulation and subsequent remodeling in damaged tissues [5]. CCL17 and CCL22 are major chemokines accounting for recruitment of $CCR4^+CD4^+$T-cells from the circulation into lesion foci [8, 11, 18, 24]. In connection with this, it is worthy that the level of serum CCL17 as well as count of peripheral blood $CCR4^+CD4^+$T-cells were increased [20].

The data of our study evidence about significantly elevated level for CCL17 and CCL22 chemokines both in acute and chronic onset sarcoidosis compared to control group. Moreover, a direct correlation between level of such chemokines and ACE concentration as a major clinical and laboratory marker of sarcoidosis activity upon chronic disease course was also noted. Such data evidence about potential use of measuring CCL17 and CCL22 level as additional parameter for sarcoidosis scoring that was also confirmed by rather high sensitivity and specificity parameters as assessed by ROC-analysis.

Nguyen CTH et al. suggest that 78% sarcoidosis patients *vs* volunteers had elevated serum TARC/CCL17 chemokine level. Subjects with higher amount of this marker were featured with more severe clinical symptoms as well as significantly higher level of markers of disease activity and severity: serum ACE level and soluble interleukin-2 receptor (sIL-2R).

Along with this, immunohistochemistry study of biopsy materials revealed that sarcoid granulomas of such patients had increased expression of $CCR4^+$T-cells and CCL17. High concentration of CCL17 was also found in patients with lesions in the three or more organs, i.e., in those having signs of the systemic disease course [18].

The data obtained evidence about marked changes in the level of examined chemokines not only in patients with more active sarcoidosis, but also in those with manifested systemic course: concentration of the chemokine CCL17 was significantly higher in patients with systemic manifestations, i.e., in subjects with organs affected along with lungs.

Signs of hepato/splenomegaly were evaluated by abdominal ultrasound examination. A number of patients had an articular syndrome, enlarged extrapulmonary lymph nodes (supraphrenic, periportal, retroperitoneal, inguinal, cervical and other localization), as well as eye damage (uveitis of sarcoid etiology).

In addition, significantly higher level of plasma CCL17 was noted in sarcoidosis patients with signs of hepato / splenomegaly. The data of ROC-analysis evidence about peak magnitude of sensitivity and specificity for CCL17 level in patients with sarcoidosis with and without splenomegaly.

Thus, measuring plasma CCL17 and CCL22 chemokines upon different clinical course of sarcoidosis contributes to deeper insight into the mechanisms of disease immunopathogenesis.

Identifying a role of $CCR4^+$T-helper cells recruited along the gradient of cognate chemokines from the peripheral blood of patients with sarcoidosis to the affected organs and tissues requires to be further investigated by using immunohistochemistry methods, which might allow to conduct a more detailed study of the mechanisms underlying granuloma formation and maturation as well as its outcomes.

To conclude, it should be noted that the peripheral blood plasma level of chemokines CCL17 and CCL22 may be used as additional prognostic markers in chronic sarcoidosis with varying scoring of clinical signs including with/without systemic disease manifestations.

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