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IMPACT OF RANTES FROM JAWBONE ON CHRONIC FATIGUE SYNDROME

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This study elucidates the question of whether chronic inflammation in the jawbone contributes to the development of Chronic Fatigue Syndrome (CFS). Fatty degenerative osteonecrosis in jawbone (FDOJ) may contribute to CFS by induction of inflammatory mediators. We examined seven cytokines by multiplex analysis in jawbone samples from two groups of patients. In order to clarify neurological interrelations, specimens from 21 CFS patients were analyzed from areas of previous surgery in the retromolar wisdom tooth area. Each of the retromolar jawbone samples showed clinically fatty degenerated and osteonecrotic medullary changes. As control, healthy jawbone specimens from 19 healthy patients were analyzed. All fatty necrotic and osteolytic jawbone (FDOJ) samples showed high expression of RANTES and fibroblast growth factor (FGF)-2. FDOJ cohorts showed a 30-fold mean overexpression of RANTES and a 20-fold overexpressed level of FGF-2 when compared to healthy controls. As RANTES is discussed in the literature as a possible contributor to inflammatory diseases, we hypothesize that FDOJ in areas of improper and incomplete wound healing in the jawbone may hyperactivate signaling pathways. Constituting a hidden source of "silent inflammation" FDOJ may represent a hitherto unknown cause for the development of CFS.

Depressive syndromes are not only associated with inflammatory diseases of the central nervous system (CNS), such as encephalitis and bacterial meningitis, but also with peripheral inflammatory processes (1). The term "sickness behavior" refers to characteristic behavioral changes that develop in the course of an infection. At the molecular level, these changes are due to the action of proinflammatory cytokines in the brain, potentially underlying the development of chronic fatigue syndrome (CFS) (2). Current research suggests that dysregulate immune functions may play a role in CFS pathogenesis. Interestingly, recent evidence points to an association of peripheral and central neuroinflammation to cytokine-chemokine networks, which may play a central role in the pathogenesis of neuropathic syndromes (3). As CFS symptoms are largely unspecific and no diagnostic test has so far been established, CFS diagnosis is often difficult and uncertain (4). The present study aims to investigate chronic inflammatory processes of the jawbone in patients with a diagnosis of CFS. The effect of fatty-degenerative osteolysis in jawbone (FDOJ) on chronic silent inflammation has been the subject of previous studies, using Luminex multi-analyte profiling technology (5). The current study addresses the question of whether FDOJ areas in CFS patients produce inflammatory messengers, activating signaling pathways that may be related to CFS development.

Key words: chemokine RANTES, chronic fatigue syndrome, osteopathy of jawbone, multiplex-analysis, signaling pathways

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MATERIALS AND METHODS

Study population

This study was performed as a randomized patient-centered controlled trial. Samples and collected data were derived directly from daily practice during normal medical treatment. Data was analyzed retrospectively. FDOJ tissue samples from 21 patients with CFS were collected. CFS was diagnosed by neurologists and physicians, based on clinical criteria for chronic fatigue syndrome. Following clinically necessary surgical FDOJ removal, conspicuous FDOJ samples of 21 CFS patients were tested for inflammatory mediators (11 cases in upper and 10 in lower FDOJ areas). All patients provided written informed consent. Patients taking any medications due to CFS complaints were not excluded from the study. Inclusion criteria were (a) therapy-resistant CFS symptomatology and (b) local diagnosis of FDOJ in edentulous jaw sites. Mandatory inclusion criteria were (c) the availability of two-dimensional orthopantomograms (2D-OPG) and (d) cone beam three-dimensional (digital volume tomograms DVT) images. A further inclusion criterion was the measurement of the bone density of the jawbone with transalveolar ultrasound technology (TAU) (8). The use of bisphosphonate medication was the main exclusion criterion for both groups. Demographic data from the CFS cohort showed an average age of 56.4 years, ranging from 30 to 79 years, and a gender ratio of 8:13 (female:male). In 12 of these patients, RANTES could also be measured in the serum in a gender ratio of 8:4 (female:male). The second cohort was a group of patients with healthy jawbone (HJB) samples, which were taken in the form of drill cores during normal dental implantation surgery. The inclusion criteria for this group were no radiologically distinctive features in 2D-OPG and inconspicuous TAU measurements of bone density in the implantation range. The age range of the control group consisting of 19 patients without FDOJ, was 38-71 years, with an average age of 54 years (SD = 12.4 years), and a gender ratio of 11:8 (female:male).

Morphology and histology of fatty degenerative samples of jawbone

The osteopathies of the bone and, in particular, the jawbone are subject to different definitions and classifications. FDOJ lesions are a phenomenon that, to date, is perceived by few specialists in medicine and dentistry. It has become increasingly apparent that intravascular coagulation plays a significant role in the pathogenesis of the osteonecrosis. The term FDOJ is used to describe a condition influenced by a wide range of factors (6). There are clear differences between FDOJ and the classical form of acute or chronic osteomyelitis. FDOJ is similar to silent inflammation or subclinical inflammation with typical signs of subliminal inflammation and painlessness. FDOJ results from an ischemic deficiency in chronic trophic disorder with fatty-degenerative spongial disintegration. Morphologically, FDOJ presents itself as greasy lumps of tissue, which are easily curetted from the medullary cavity of the jawbone (Fig. 1). Bouquot et al. described FDOJ-induced necrotic and cancellous bone as follows: "Hollow cavities containing soft tissue that has undergone fatty dystrophic changes and delamination of the bony sheath of the inferior alveolar nerve" (6). Fig. 1 shows such a specimen with the predominantly fatty transformation of the jawbone (left panel). The often impressive extent of FDOJ lesions is indicated by the contrast agent in the X-ray image (right panel), which was introduced immediately after surgical FDOJ curettage and removed following the X-ray.

A characteristic definition of FDOJ can be inferred from over a thousand histological findings at the corresponding author's clinic. Notably, the number of fat cells is consistently and strikingly increased in all FDOJ samples. Typical signs of inflammation, especially that of an inflammatory cell response, are absent. The histologic examination of the curetted tissue demonstrates ischemia, necrotic adipocytes, and myxoid degeneration. Aseptic, ischemic osteonecrosis (AIO) appears as the type of bone lesion that most closely resembles FDOJ (7). According to previous research (6), FDOJ represents lesions similar to those found in long bones primarily defined as "bone marrow edema" and "chronic nonsuppurative osteomyelitis".

Sampling of FDOJ tissue

The current treatment of FDOJ lesions consists of curettage of the bony cavity. To elucidate a possible causative link between FDOJ and CFS at the Munich Clinic for Integrative Dentistry, Germany, 21 patients with CFS who were diagnosed with FDOJ had surgery on the affected area of the jaw. After local anesthesia and

folding back of a mucoperiosteal flap, the cortical layer was removed. All 21 patients exhibited FDOJ inside the bone marrow, similar to the samples described in the literature (5-7).

Processing of necrotic tissue samples and multiplex analysis

The analysis was taken from 40 samples of jawbone: 21 samples of FDOJ in patients with CFS plus 19 healthy jawbone samples as a control group. At the Institute for Medical Diagnostics, Berlin, inspected by DAKKS (Deutsche Akkreditierungsstelle GmbH, accredited to DIN EN ISO/IEC 17025:2005 and DIN EN ISO 15189:2007), the samples were homogenized by mechanical force in 200 µL of cold protease inhibitor buffer (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics GmbH, Penzberg, Germany). The homogenate was then centrifuged for 15 min at 13,400 rpm. Next, the supernatant was collected and centrifuged for further 25 min at 13,400 rpm. In the 15 supernatants of tissue homogenate, regulated on activation, normal T-cell expressed and secreted (RANTES), also known as chemokine C-C motif ligand 5 (CCL5), FGF-2, interleukin- (IL-)1 receptor antagonist (ra), IL-6, IL-8, monocyte chemotactic protein-1 (MCP1), and tumor necrosis factor-alpha (TNF-α) were measured. The measurement was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions and analyzed using Luminex 200 with xPonent software (Luminex Co, Austin, TX, USA).

Analysis of RANTES in serum

In 14 patients of the CFS-FDOJ cohort, RANTES was additionally analyzed in serum. The measurement was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K, Millipore GmbH, 65824 Schwalbach, Germany) according to the manufacturer's instructions and analysed using Luminex® 200™ with xPonent® Software (Luminex, Austin, TX, USA). For the measurement of RANTES in serum, samples were pre-diluted 1:100 in sample buffer according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using IBM SPSS, version 19 (IBM Corporation, Armonk, NY, USA). All data was presented as a mean ± standard mean error. The two-sided unpaired *t*-test was used in determining the differences in the groups while a Spearman coefficient was used in examining the correlation presence among the cytokines analyzed. Data was considered significant where the value was <0.05.

RESULTS

Immune mediators were analyzed in FDOJ samples from CFS patients (n=21) and in drilled explants from normal jawbone of healthy controls (n=19). This comparison showed significantly elevated levels in FDOJ samples versus control tissue for fibroblast growth factor (FGF-2; 762±314 *vs* 27.6±36, $p<0.0001$), interleukin 1 receptor antagonist (IL1-ra; 718 ±435 *vs* 197±569, $p<0.01$), monocyte chemoattractant protein 1 (MCP-1; 123±205 *vs* 20.3±20.5, $p<0.05$), and regulated on activation, normal T-cell expressed and secreted / C-C-chemokine ligand 5 (RANTES/CCL5; 5199±2648 *vs* 150±131 $p<0.0001$). At the same time, there was a significant decrease of interleukin 6 (IL-6; 5.4±14.7 *vs* 101±81.7, $p<0.0001$). No significant differences were observed for interleukin 8 (IL-8; 12.6±15.5 *vs* 7.5±3.9, $p= 0.17$) and tumor necrosis factor alpha (TNF-alpha; 5.3±23.3 *vs* 11±3.2, $p=0.3$). Reference values for healthy jawbone tissue were not available in the literature.

Based on these distributions, we focused our study on the highly overexpressed chemokine RANTES being the most significantly overexpressed signaling pathway in further discussion. Overall, the changes in cytokine distribution between healthy and FDOJ tissue show a prominent increase in RANTES with a concomitant decrease of acute cytokines IL-6 and (by non-significant trend) TNF-alpha (Fig. 2).

In an effort to address any possible effects of RANTES expression in FDOJ on the pathogenesis of CFS, we examined RANTES serum levels in the CFS cohort. Evaluation of 13 CFS patients available for serum sampling revealed a mean RANTES level of 42.6±17.2 ng/mL, with 11 of 13 patients exceeding the published normal range of < 29 ng/mL (9). Comparison of matched serum and FDOJ samples indicated a significant correlation of RANTES tissue

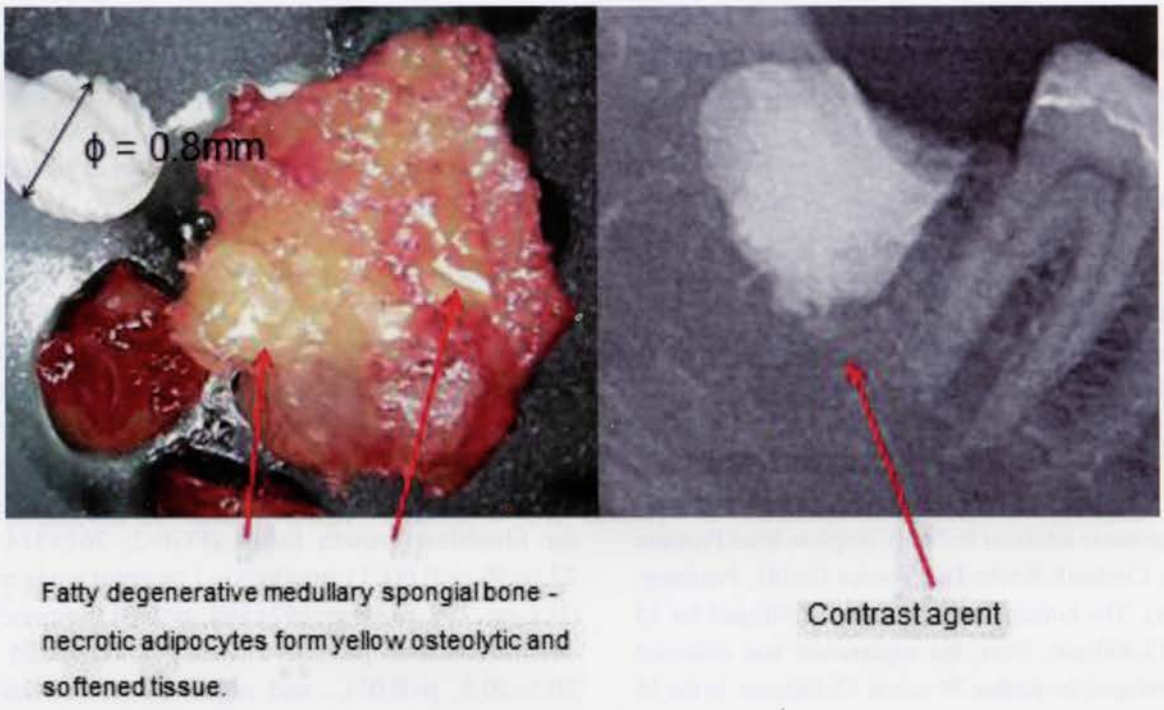

Fig. 1. *FDOJ sample of fatty and osteolytic degenerated bone marrow; head of the drill with 0.8 mm diameter shows the extent of FDOJ (left panel) and contrast medium in FDOJ cavity after curettage (right panel).*

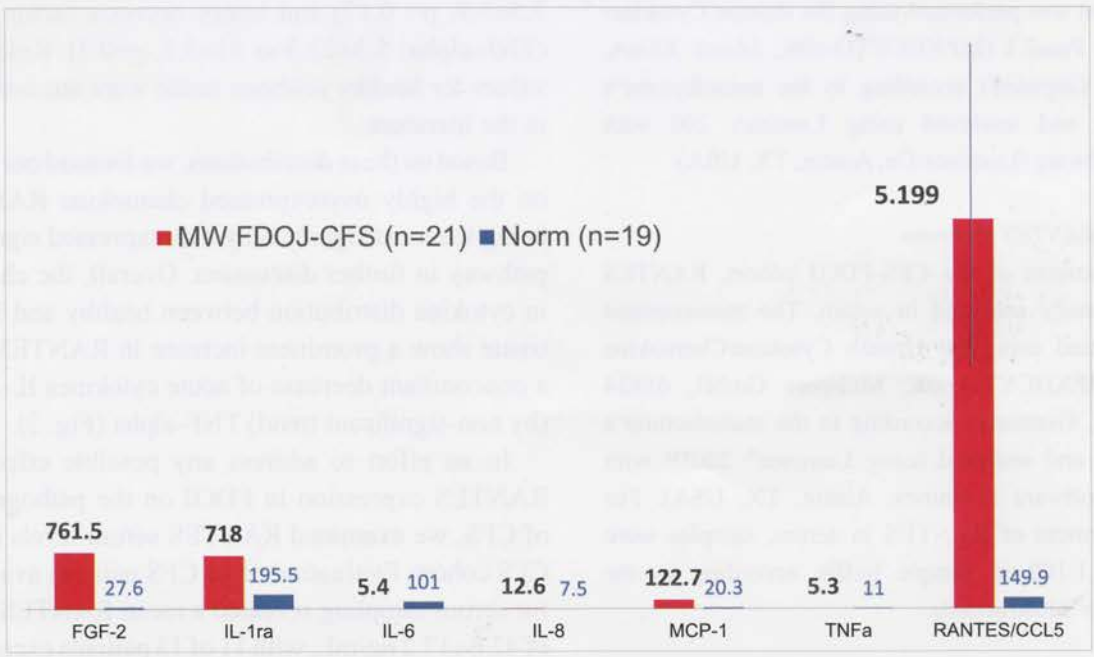

Fig. 2. *Distribution of 7 cytokines in healthy jawbone (blue columns/ n = 19) vs in FDOJ specimens of the CFS cohort (red columns/n = 21) in pg/ml.*

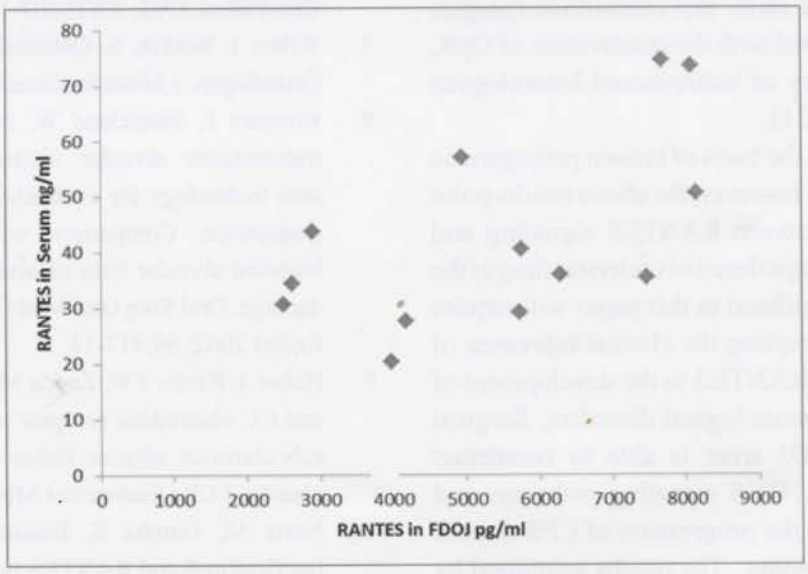

Fig. 3. *RANTES levels in serum from the CFS cohort (n=13) compared to RANTES levels in FDOJ.*

levels in FDOJ to serum levels ($r=0.59$, $p<0.05$), pointing to FDOJ tissue as a source for the increase of systemic RANTES (Fig. 3).

DISCUSSION

Our data show increased RANTES levels both in FDOJ tissue and the serum of CFS patients. Considering the potential systemic effects of elevated RANTES levels we hypothesize that RANTES induced immunological and neurological changes contribute to CFS pathogenesis, rendering FDOJ a hitherto unrecognized risk factor for CFS. In obese patients RANTES promotes ischemia by reducing blood flow and capillary density (9, 10). Recent data show that ischemia associated chemokine expression increases local and systemic secretion of inflammatory cytokines (11). CFS is associated with elevated levels of inflammatory cytokines, suggesting that cytokines play a role in CFS pathophysiology (12). By secretion of RANTES, FDOJ lesions may thus initiate inflammatory pathogenesis, leading to the development of chronic fatigue. Inflammatory cytokines are able to penetrate the blood-brain-barrier and bind to specific receptors, inducing behavioral changes that are referred to as sickness behavior in animal models (13). At the same time, chemokines such as RANTES facilitate leukocyte trafficking across the blood-brain barrier, promoting central inflammatory processes (14). Immunohistochemical studies have shown that RANTES and other chemokines are present in several brain regions such as the hypothalamus, the limbic system and the hippocampus (15). Direct receptor binding of RANTES has been demonstrated for both neurons and astrocytes (16). In addition to their proinflammatory effects and chemotactic functions, chemokines have been implicated in various neurobiological processes potentially relevant to psychiatric disorders, such as neuromodulatory effects, neurotransmitter action and regulation of neurogenesis (17). It is intriguing to speculate that chemokines may constitute a third transmitter system in the brain, regulating brain function by interaction with both neurotransmitters and neuropeptides. Moreover, recent studies have suggested that RANTES modifies nociception by direct interaction with opioid receptors (OpR) (18), promoting receptor desensitization. As OpR signaling induces analgesia, RANTES-mediated desensitization leads to increased nociception (19).

Current *in vitro* data show that chemokine receptor activation is associated with the inactivation of OpR, supporting the theory of bidirectional heterologous desensitization (20, 21).

In conclusion, on the basis of known pathogenetic mechanisms of CFS, however, the above results point to an interaction between RANTES signaling and CNS functions. A comprehensive understanding of the complex networks outlined in this paper will require further research addressing the clinical relevance of chemokines such as RANTES to the development of CFS and psycho-immunological disorders. Surgical debridement of FDOJ areas is able to counteract the induction of RANTES signaling pathways and thus possibly reduce the progression of CFS disease and associated symptoms. The results submitted by the authors are able only to highlight the need for a large multi-center prospective study with clearly defined cohorts, to clarify causal backgrounds to the development of CFS.

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