FOLLOW-UP OF PATIENTS WITH SYSTEMIC IMMUNOLOGICAL DISEASES UNDERGOING FATTY-DEGENERATIVE OSTEOLYSIS OF THE JAWBONE SURGERY AND TREATED WITH RANTES 27CH

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Regulated-on-activation, normal T cell expressed and secreted (also called RANTES, CCL5 or R/C) is a chemotactic cytokine that plays a key role in recruiting immune cells to inflammatory sites. R/C is involved in the pathogenesis of many systemic immune-mediated diseases (SIDs) and is upregulated in fatty-degenerative osteolysis jawbone (FDOJ) cavitations. Surgical cleaning of degenerative areas reduces the source of chronic R/C but might not be sufficient to reestablish the altered immunological patterns. The aim of the present study was to collect clinical data from patients suffering from SIDs who underwent dental surgery of FDOJ areas (n=46), by measuring R/C serum levels at the first visit (V0) prior to surgery, and at the second visit (V1). The majority of patients (n=41) were treated one month with ultra-low dose RANTES (27CH), a medicine used in micro-immunotherapy, while five patients were not. Mean and standard deviation of R/C serum levels at V0 in treated and untreated patients were respectively 48.5±25.8ng/ml and 42.48±22.22ng/ml. Untreated patients had a tendency towards higher R/C levels at V1 (68.36±30.7ng/ml; p=0.062), while an opposite tendency was observed in treated patients (40.9±20.3ng/ml; p=0.129). Investigators observed that a cut-off set at 40ng/ml at V0 seemed to be predictive of the efficacy of the dental surgery/treatment (p=0.0013, n=26) and that gender could influence R/C levels and patient's responsiveness. The Authors, being aware that this is a preliminary follow-up, wanted to lay the basis for forthcoming studies, in which a larger cohort of patients and well-defined inclusion/exclusion criteria will be established.

Regulated-on-activation, normal T cell expressed and secreted (also called RANTES, CCL5 or R/C) is a chemotactic cytokine that plays a key role in recruiting leukocytes, macrophages and eosinophils to inflammatory sites. Together with other inflammatory cytokines, R/C is implicated in the formation of inflammatory infiltrates. While most of the chemokines are expressed during the 'immediate early response', R/C is expressed 3–5 days after T-cell activation (1). R/C is involved in broad inflammatory features and in the pathogenesis of a variety of systemic immune-mediated diseases (SIDs), including cancer, atherosclerosis, neurological diseases, rheumatoid arthritis, diabetes, and Hashimoto's thyroiditis (2-4). SIDs cover a wide range of systemic disorders, directly and indirectly immune-related, in which immunological components, such as R/C and its receptors, can be

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used as therapeutic targets. Indeed, therapeutic strategies aiming at decreasing R/C expression could be used to treat atherosclerosis (4), dysfunctions after transplantation (5) and autoimmune diseases (6, 7).

Jawbone cavitations (JCs) are jawbone areas characterized by dying or dead bone marrow. These fatty degenerative cavitations may be painful or, in some cases, can remain asymptomatic for years. JCs which are affected by fatty-degenerative osteolysis of the jawbone (FDOJ) may be distinguished by the overexpression of inflammatory mediators, and more specifically of R/C (8-10). The chronic R/C input might influence the immunological pattern and aggravate systemic immunological diseases (9, 10). Nowadays, the existence of FDOJ is largely ignored in mainstream dentistry. The reason is that conventional X-ray techniques have limited ability to visualize FDOJ areas (11) and, because of these diagnostic difficulties, jawbone disease is often underdiagnosed by dentists. To give the practitioner an aid to the diagnosis, a computer-assisted through-transmission alveolar ultrasound device was developed (12). Surgical cleaning of the FDOJ areas are currently aimed at reducing the source of chronic R/C input by removing the adipocytes from the inflamed JCs areas. However, the surgical removal of R/C source might not be sufficient to reestablish the altered immunological patterns, especially when patients suffer from immune-mediated diseases. In these patients, surgery could be improved by modulating the inflammatory pathways and, more specifically, by decreasing circulating R/C levels.

Immune system imbalances might be restored using a micro-immunotherapy (MI) approach. MI is a therapeutic strategy which uses immune regulators, including cytokines, in association with nucleic acids (plant-derived total DNA and RNA) and specific nucleic acids (SNA®). The active substances, prepared in ultra-low doses, are used in sequential formulas developed to treat different acute and chronic diseases. The use of RANTES 27CH, aims at reducing the altered levels of R/C and supports recovery after dental surgery of FDOJ areas. The goal of this follow-up is to evaluate the impact of RANTES 27CH in patients suffering from systemic immunological diseases, undergoing dental surgery of FDOJ areas, by measuring R/C serum level before surgery (first visit, V0) and after one month of RANTES 27CH intake, at a second visit, V1.

MATERIALS AND METHODS

Patient characteristics

The research is based on data retrieved from 46 patients who underwent dental surgery, of whom 41 patients took the RANTES 27CH treatment for one month, while five did not. The patient characteristics are shown in Table I. All but three patients suffered from SID and chronic inflammation: chronic fatigue syndrome (CFS) (n=10), breast cancer (BC) (n=5), multiple sclerosis (MS) (n=5), rheumatoid arthritis (RA) (n=10), trigeminal neuralgia (TN) (n=8), etc. (Table I). Due to the different nature of SIDs and the treatment strategies, no exclusion criteria were listed. All patients gave their written informed consent to the surgery, the blood collection for further analysis and to medication intake. Blood samples were collected from all patients: before FDOJ surgery (first visit, V0) and after 20 to 526 days (second visit, V1).

Laboratory analysis

Blood samples were collected before surgery (V0) in S-Monovette 7.5-ml Z tubes, clot activator Silica (Fa. Sarsted AG & Co. Nümbrecht, Germany). Once collected, blood was swiveled at least 2 times, then stored at 4°C and sent the same day to the laboratory that performed the analysis. The same procedure was carried out for treated and untreated patients at the second visit (V1). Measurement of RANTES/CCL5 in serum was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions and analyzed using the Luminex® 200TM with xPonent® Software (Luminex Co, Austin, TX, USA). For measurement of RANTES/CCL5 in serum, samples were prediluted 1:100 in sample buffer according to manufacturer's instructions. To avoid dilution factor error, two control samples (low and high) were run in parallel and the two controls variance was calculated as well as the measurement fluctuations. The mean squared deviation for controls was below 10% (9.5% for low and 7.0% for high).

The analyses were carried out 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]).

Treatment and dosing

RANTES 27CH is a micro-immunotherapy medicine manufactured by Labo'Life, and notified to the Belgian Federal Agency for Medicines and Health Product (FAMHP) under notification number 1507UH150F33. The medicine consists of lactose-saccharose globules impregnated with an ultra-diluted ethanolic preparation of RANTES, obtained by pharmacopeial dilution/succussion steps, reproduced 27 times. The globules, packed into capsules, are sublingually taken in the morning, on an empty stomach. The posology for the 41 patients was of one capsule per day, during one month.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 7.0. R/C serum levels at V0 were compared to post-treatment levels (V1) using paired 2-tailed Student's *t*-tests. If data were not normally distributed Wilcoxon matched pair test was preferred. The unpaired *t*-test was performed to analyze differences in RANTES levels at V0 between men and women. 0.05 was considered as p-value threshold to assess statistical significance.

RESULTS

Of the five control patients who underwent FDOJ surgery without taking RANTES 27CH, all presented higher levels at V1 compared to V0. Due to the small number of patients, normality test failed and the difference came out as not statistically significant (Wilcoxon test, p=0.062, n=5). Mean and standard deviation were 42.48±22.22ng/ml at V0 and 68.36±30.7ng/ml at V1 (Fig. 1A). Analyzing R/C serum levels in patients treated with RANTES 27CH, an opposite effect was observed, with a tendency towards lower levels at V1 compared to V0 (Wilcoxon test, p=0.129, n=41). The mean and standard deviation at V0 and V1 were respectively 48.5 ng/ml±25.8 and 40.9 ng/ml±20.3 (Fig. 1B). In order to evaluate whether the time spent between the first and the second visit could influence results and conclusions of the follow-up, the patients were grouped in three windows of time, based on the number of days after the first visit (V0) as follow:

Table I. *Characteristics of patients.*

VARIABLE	FDOJ patients without RANTES 27CH (n=5)	FDOJ patients treated with RANTES 27CH (n=41)
Sex	M (n=2); F (n=3)	M (n=16); F (n=25)
Chronic fatigue syndrome (CFS)	M (n=0); F (n=0)	M (n=8); F (n=2)
Breast cancer (BC)	F (n=0)	F (n=5)
Multiple sclerosis (MS)	M (n=0); F (n=1)	M (n=1); F (n=3)
Rheumatoid arthritis (RA)	M (n=1); F (n=1)	M (n=4); F (n=4)
Allergy	M (n=1); F (n=0)	M (n=0); F (n=1)
Hashimoto's thyroiditis	M (n=0); F (n=0)	M (n=0); F (n=1)
Migraine	M (n=0); F (n=0)	M (n=0); F (n=1)
Trigeminal neuralgia (TN)	M (n=0); F (n=1)	M (n=3); F (n=4)
Osteoporosis	M (n=0); F (n=0)	M (n=0); F (n=1)
No SID	M (n=0); F (n=0)	M (n=1); F (n=2)
Days after V0 (V1)	35 - 160	20 - 526

between 20 to 50 days, between 51 and 80 days, and more than 80 days. Only in those patients having their follow-up visit (V1) within 50 days, a significant reduction of the levels of RANTES was observed at V0 compared to V1 (paired *t*-test, p=0.034, n=15) (Fig. 1C). When visit V1 occurred after 50 days, being between 51 and 80 days, and between 81 and 526, no significant changes were observed in serum R/C levels (data not shown). Findings suggest that FDOJ patients treated with RANTES 27CH, checked between 20 and 50 days post-surgery, responded as expected to the treatment and dental surgery.

Having listed the R/C concentration at V0 in ascending order, a cut-off was identified at 40 ng/ml; patients who had R/C serum values at V0 over 40 ng/ml responded as expected to the surgery and treatment with a reduction of their R/C levels at V1 (Wilcoxon test, p=0.0013, n=26). On the other hand, serum levels of the patients who had values at V0 below 40 ng/ml, increased (Fig. 2, A, B).

Considering that patients were treated for one month, they were further grouped into those having their R/C levels at V0 below and over 40 ng/ml, based on the days passed after V0 to analyze the three windows of time: between 20 to 50 days, between 51 and 80 days, and more than 80 days. The increase of the R/C serum levels at V1, in patients with their levels at V0 <40 ng/ml, was not confirmed within the 50 days after V0, nor in the two other groups of patients (Fig. 2C). On the other hand, the responsiveness described in patients having R/C levels at V0 >40 ng/ml, was confirmed in the subgroup of patients who had their second visit within 50 days (paired *t*-test, p= 0.0057, n=10) (Fig. 2D).

Because no significant differences exist between men and women in RANTES levels at V0 (unpaired *t*-test, p=0.19), nor in the days after the first visit (unpaired *t*-test, p=0.23), it was analyzed whether gender could influence the responsiveness to the treatment/dental surgery. As shown in Fig. 3A, the R/C levels in men at V1 were lower than levels at V0 (Wilcoxon test, p=0.0083, n=16), while no changes were observed in women. Again, the reduction of serum R/C levels was observed only in men having their V0 levels higher than 40 ng/ml, as shown in Fig. 3B (Wilcoxon test, p=0.0002, n=13). No changes

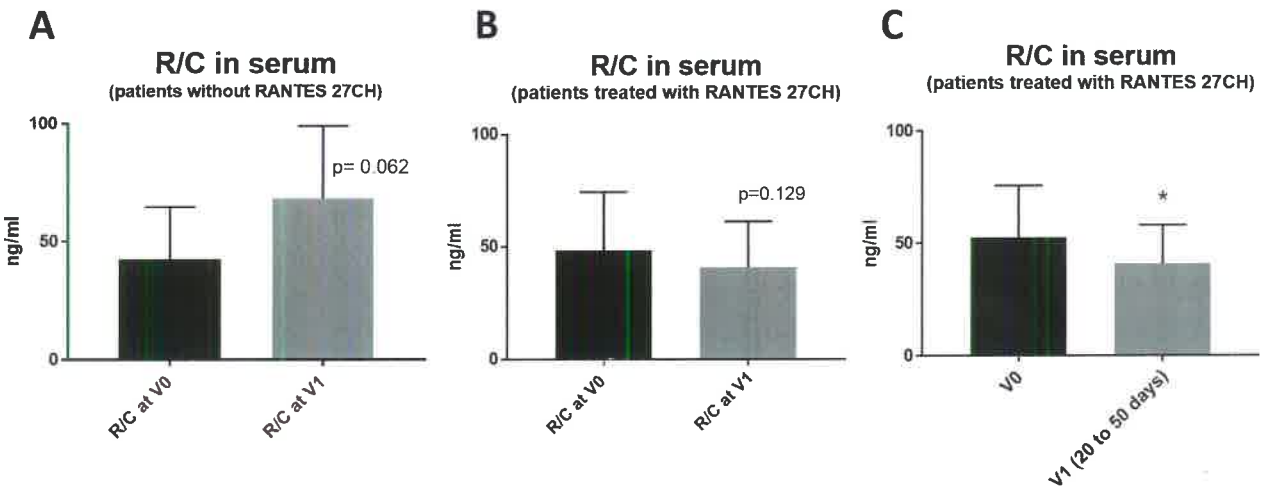

Fig. 1. *RANTES levels in FDOJ patients treated or not with RANTES 27CH.* ***A)*** *R/C serum levels (ng/ml) measured in FDOJ patients at first visit V0, before surgery, and at second visit V1 (N=5). There is a trend towards higher R/C levels at V1 compared to V0.* ***B)*** *R/C serum levels (ng/ml) measured in FDOJ patients at first visit V0, before surgery, and at second visit V1, after one month of treatment with RANTES 27CH (N=41). There is a trend towards lower R/C levels at V1 compared to V0.* ***C)*** *R/C serum levels (ng/ml) measured in FDOJ patients at first visit V0, before surgery, and at second visit V1, performed within 50 days, after one month of treatment with RANTES 27CH. R/C levels decreased at V1 (p=0.034, n=15). Error bars represent standard deviation.*

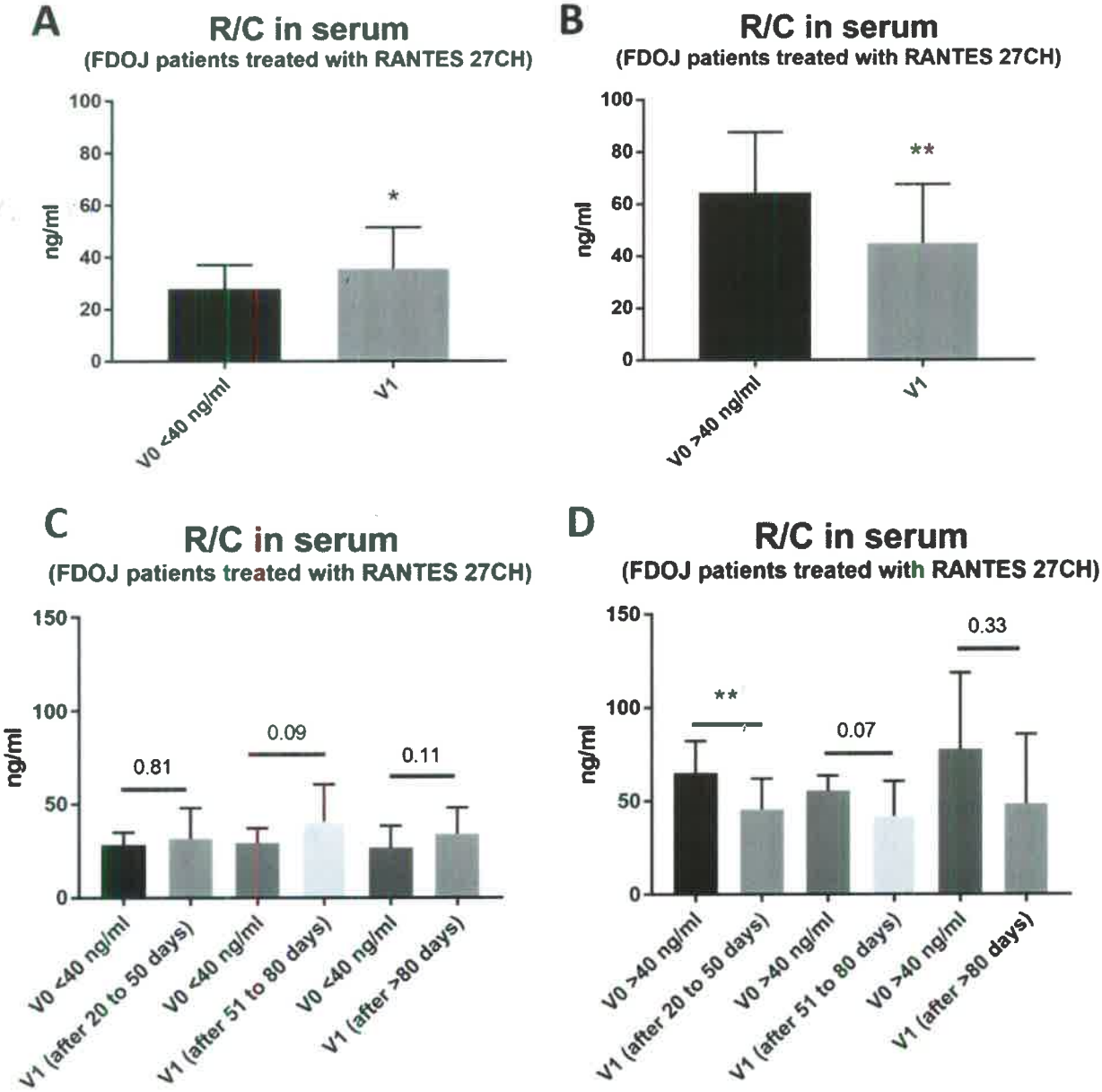

Fig. 2. *Treatment response cut-off at 40 ng/ml at V0.* ***A)*** *R/C serum levels (ng/ml) measured in FDOJ patients having their levels below 40 ng/ml at first visit V0, and at second visit V1, after one month of treatment with RANTES 27CH. R/C levels increased at V1 compared to V0 (p=0.017, n=15).* ***B)*** *R/C serum levels (ng/ml) measured in FDOJ patients having their levels over 40 ng/ml at first visit V0, and at second visit V1, after one month of treatment with RANTES 27CH. Serum R/C levels at V1 decreased compared to V0 (p=0.0013, n=26).* ***C)*** *R/C serum levels (ng/ml) measured in FDOJ patients having their levels at V0 below 40 ng/ml treated with RANTES 27CH for one month, and having their second visit V1, within 50 days, between 51 and 80 days, and at more than 80 days. No significant changes were observed in R/C levels at V1 compared to V0.* ***D)*** *R/C serum levels (ng/ml) measured in FDOJ patients having their levels at V0 over 40 ng/ml, treated with RANTES 27CH for one month, and having their second visit V1, within 50 days, between 51 and 80 days, and at more than 80 days. Only in those patients having their V1 within 50 days, authors observed a reduction in serum R/C levels (p=0.0057, n=10). Error bars represent standard deviation.*

were seen of circulating R/C levels between V0 and V1, in women patients grouped by R/C serum levels at V0: below or over 40 ng/ml (Fig. 3C).

Changes in R/C levels were also examined at V1 versus V0 by grouping the patients by gender and SID. As shown in Fig. 4, a tendency toward an R/C level reduction at V1 compared to V0 was observed in BC patients (Wilcoxon test, p=0.06, n=5) and in male patients affected by CFS (paired *t*-test, p=0.16, n=10), TN (Wilcoxon test, p=0.25, n=7) and RA (Wilcoxon test, p=0.21, n=8). However, the small number and the heterogeneity of patients

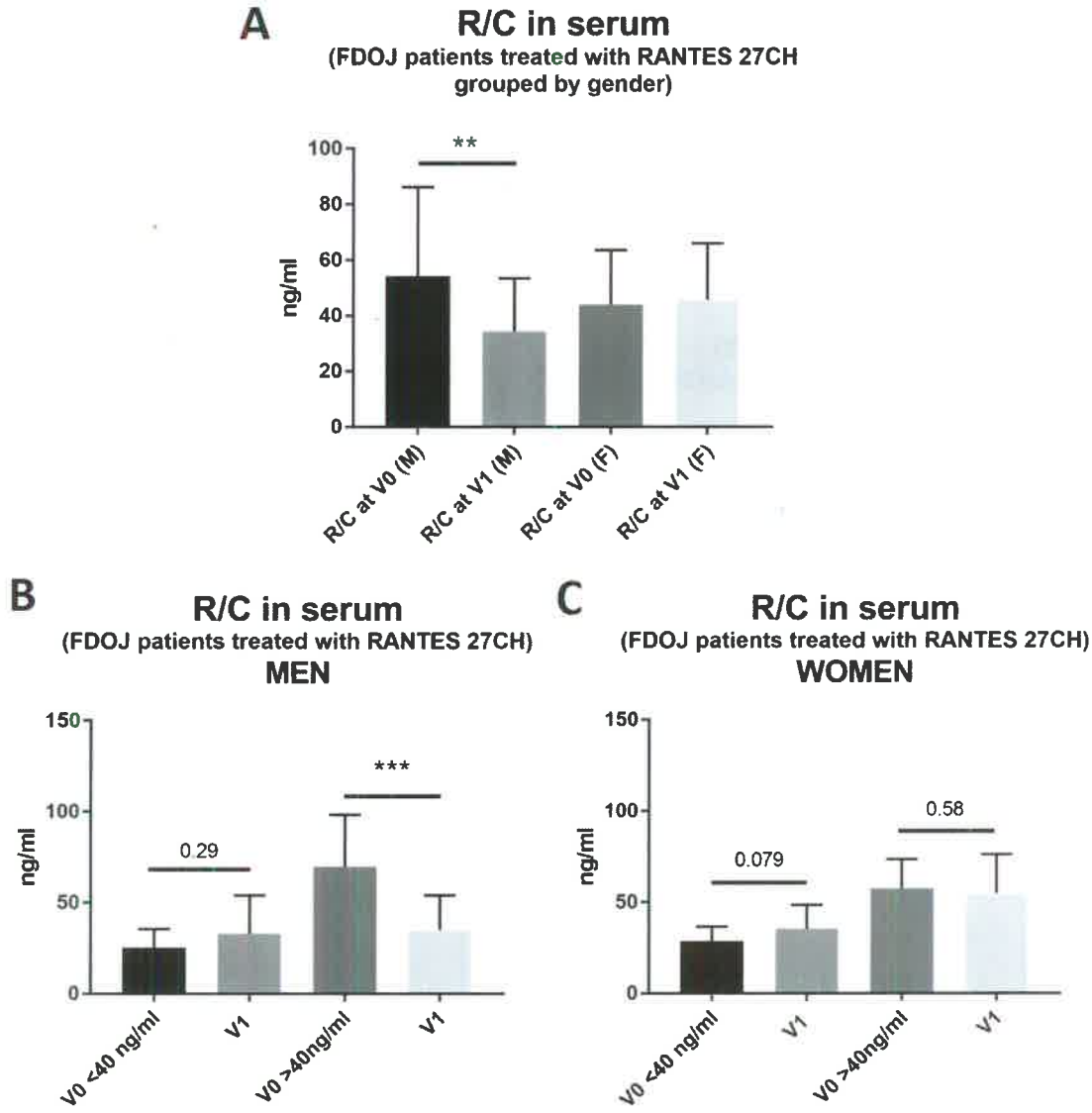

Fig. 3. *Gender and cut off at 40 ng/ml at V0 were considered to group the treated patients. **A)** R/C serum levels (ng/ml) measured in FDOJ patients (men and women) treated with RANTES 27CH for one month. Men responded with lower levels at V1 compared to V0 (p=0.0083, n=16). No significant changes were observed in R/C levels at V1 compared to V0 in women. **B)** R/C serum levels (ng/ml) measured in FDOJ patients (men) treated with RANTES 27CH for one month, having their levels at V0 below or over 40 ng/ml. Only in those patients having levels >40 ng/ml, authors observed a reduction in serum R/C levels (p=0.0002, n=13). **C)** R/C serum levels (ng/ml) measured in FDOJ patients (women) treated with RANTES 27CH for one month, having their levels at V0 below or over 40 ng/ml. No significant changes were observed. Error bars represent standard deviation.*

participating in the follow-up made it difficult to draw any conclusions.

DISCUSSION

Dental surgery allows the elimination of the local source of R/C, however, it may not be sufficient to reduce circulating levels of R/C, especially in those patients suffering from other pathologic conditions that can affect directly and/or indirectly chemokines and R/C expression.

In the described cohort of FDOJ patients, R/C levels were all higher than baseline levels reported by Biancotto et al. in healthy subjects. In the cited study, the Authors analyzed serum levels of various cytokines, including RANTES, using a Bio-Rad 27-plex Luminex kit. Mean of RANTES in men and in women were respectively 9534.5 pg/ml (=9.5 ng/ml) and 11848.9 pg/ml (=11.8 ng/ml) (13). Quantification of cytokines varies significantly depending on sample collection procedures, commercial kit performance, other pre-analytical variables, and it is difficult to make a comparison among clinical studies. For those reasons, the present Authors are prudent in affirming that the circulating levels of RANTES found in FDOJ patients are higher than the healthy baseline levels.

In order to reduce the inflammation in FDOJ patients and, in particular, to decrease the circulating

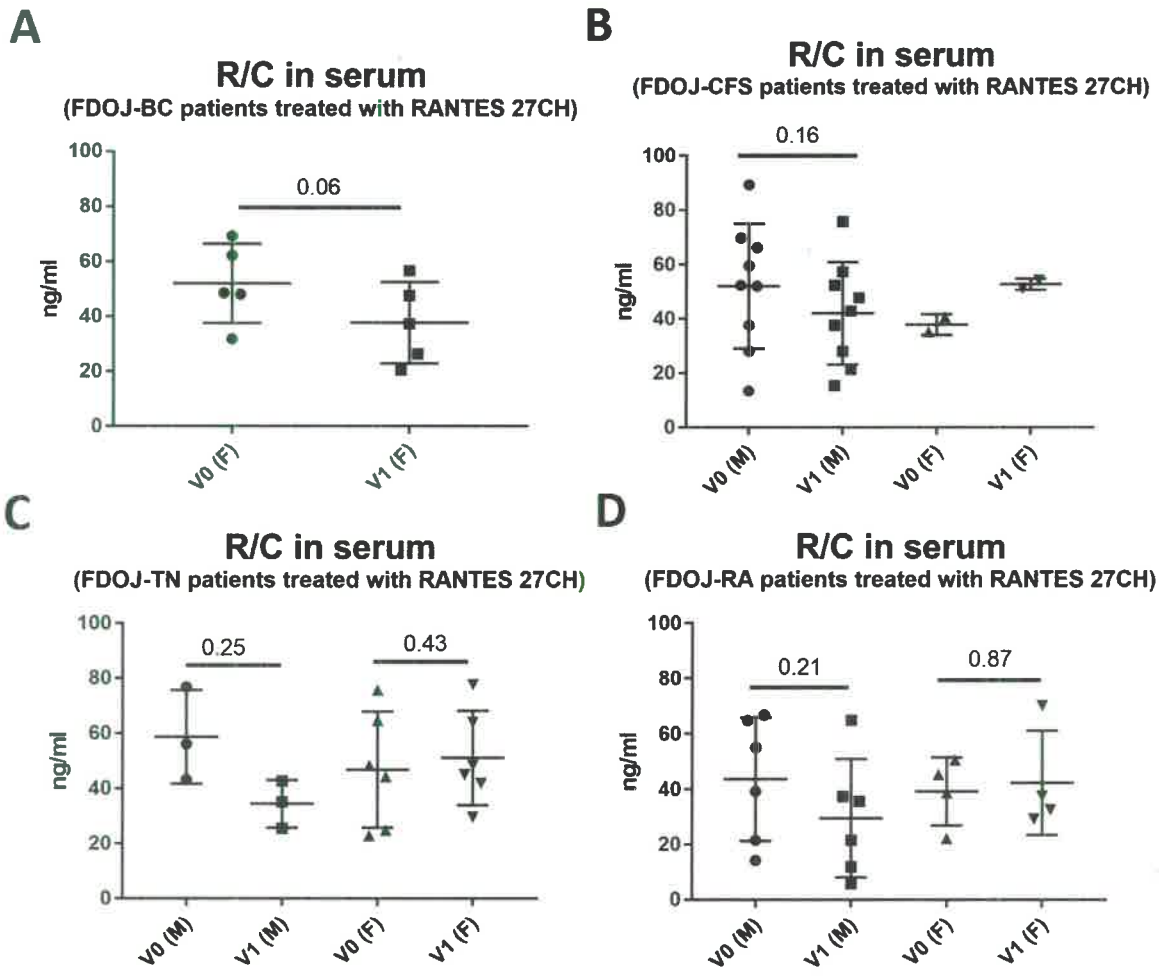

Fig. 4. *Patients were grouped by gender and type of SID to check differences in treatment response. Scatter plot (mean and standard deviation) representing R/C serum levels (ng/ml) measured in FDOJ patients (men and women) treated with RANTES 27CH for one month, affected by (**A**) breast cancer (BC), (**B**) chronic fatigue syndrome (CFS), (**C**) trigeminal neuralgia (TN), and (**D**) rheumatoid arthritis (RA).*

R/C level, most of the patients were treated during one month (n=41) with the ultra-low dose chemotactic cytokine RANTES 27CH, while five patients were not. The ultra-diluted medicine RANTES 27CH aims at reducing circulating levels of RANTES inducing a "hormetic response". The term "hormesis" consists of the biphasic dose phenomenon, firstly observed by the pharmacologist and toxicologist Schulz, during his multiple chemical tests on yeast, and further studied by Calabrese (14). Hormesis is a cellular response that gives the ability to correct an altered homeostasis, adjusting to conditions that are optimal for cell survival; hormesis is the plausible mode of action of homeopathic medicines (15) and the possible mechanism of action of ultra-diluted medicines, including RANTES 27CH.

The five untreated FDOJ patients had their R/C serum levels increased at V1, while on the other hand, patients treated with RANTES 27CH tended to lower levels at V1 compared to V0. Analyzing differences in treatment responses, the Authors observed: i) a response cut-off at 40 ng/ml at V0; ii) that the patients having their second visit within 50 days responded as expected to the dental surgery/ treatment with decreased R/C levels at V1 compared to V0 ($p=0.0057$, $n=10$); iii) that only men having their levels at V0 over 40 ng/ml had their levels decreased at V1 ($p=0.0002$, $n=13$). The gender difference could be attributed to the fact that in women, the difference in estrogen, progesterone, or both hormone levels, may have influenced R/C expression and, in turn, the surgery/treatment responsiveness.

In conclusion, this preliminary follow-up of SID patients who underwent FDOJ surgery, suggests that R/C serum level over 40ng/ml as well as gender could influence the responsiveness of the dental surgery followed by the anti-inflammatory medicine RANTES 27CH intake. These findings should be confirmed in a larger cohort of patients in which well-defined inclusion/exclusion criteria would be established.

Conflict of interest

JL has no conflicting commercial or financial interests. IF and BL work for Labo'Life, the pharmaceutical company that develops and commercialized micro-immunotherapy medicines, including RANTES 27CH. This professional relationship does not influence the scientific neutrality in regard to this research.

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