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Adjuvant benefit of a peptide-rich marine biology formula (LD-1227) in rheumatoid arthritis: a randomized, double-blind, controlled study

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ABSTRACT

Introduction. There is a growing interest on non-chemical therapies among patients suffering from rheumatoid arthritis (RA), although safety, efficacy and properly designed studies are often lacking.

Objective. The aim of the present investigation was to explore the clinical effectiveness of a marine nutraceutical, LD-1227, endowed by fine molecular biology studies, in the management of RA.

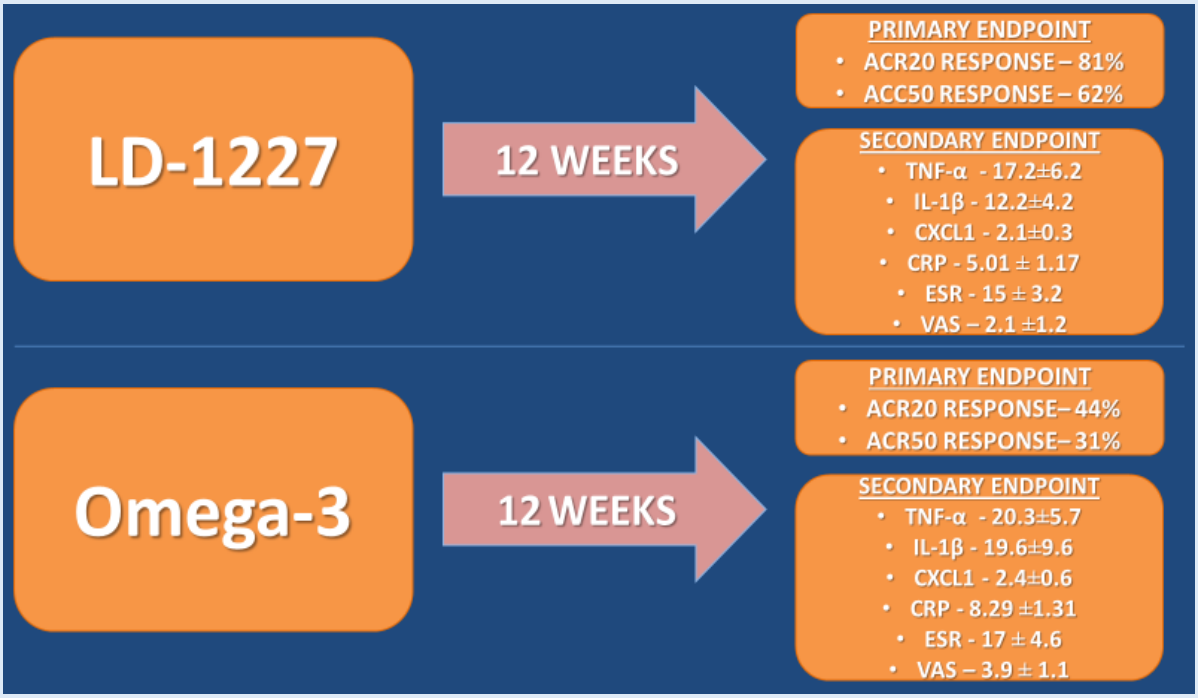
Methods. The study design was a 12-week, randomized, double-blind study involving forty patients with stable long-standing RA who were randomized to receive either LD-1227 (n = 20) or Omega-3 (n = 20) on top of their established maintenance therapy.

Results. At study recruitment and after 12 weeks of treatment, their Health Assessment Questionnaire (HAQ), erythrocyte sedimentation rate (ESR), visual analogue scale (VAS), and Disease Activity Score (DAS) 28, anxiety and depression analysis, C-reactive protein (CRP) levels, CXCL1, several pro-inflammatory interleukins levels and related gene expression, were compared between the two groups. Primary end point was the proportion of patients with response at weeks 12 as from the 20 % to 50% improvement criteria of the American College of Rheumatology (ACR20). At 12 weeks, ACR20 beneficial response was 81.0 % in LD-1227 group and 44 % in omega-3 group, ($p< 0.01$). The superiority of LD-1227 appeared also when considering the ACR50 response at 12 weeks (62% in LD-1227 group as compared to 31 %

in omega-3 group, p< 0.01). The LD-1227-treated group displayed a significant improvement of VAS scale, HAQ score, morning stiffness and tender points (p < 0.01 vs control and p < 0.05 vs omega-3, respectively). From the biochemical viewpoint, patients in the LD-1227 group showed a lower level of CRP, IL-6, TNF-α, IL-1β, CXCL1, IFNγ, IL-15 and IP-10 and significant downregulation of related gene expressions. Unlike what observed in LD-1227 group, in the omega-3 group, CRP and DAS28 did not reach statistical difference. A substantial reduction of extra pain killer use was noted under LD-1227 treatment.

Conclusion. One can conclude that LD-1227 may play a significant role on the management of RA and with a specrum and mechanisms of actions distinct from the canonical omega-3 while being devoid of any side effect or tolerability issues.

Keywords: rheumatoid arthritis, cytokines, omega-3, marine peptides, inflammation, pain, gene expression



INTRODUCTION

Rheumatoid arthritis (RA) is a common, long-term inflammatory autoimmune disease, which affects 0.5 to 1% of adults in the developed world with 5 to 50 per 100,000 newly developed cases each year [1]. This is characterized by cartilage derangement with ongoing synovitis, and joint disabilities often in a “symmetrical” pattern significantly impairing quality of life [2, 3]. This condition brings about a 10-15 year shortening of life expectancy as compared to the overall population besides a considerable economic burden [4]. RA mainly affects the elderly, with about 30% of patients experiencing initial discomfort after the sixth decade [5]. Cartilage erosion following relentless synovial inflammation is the hallmark of the disease. In particular, fibroblast-like synoviocytes in RA subjects show hyperproliferative changes with apoptosis inhibition due to the activation of transcription 3 (STAT3) signaling. Ultimately this tends to perpetuate these leading to cartilage and bone erosion [6, 7]. The concomitant

inflammatory cytokines release, namely IL-6, by binding to their receptors trigger Janus kinase 2 (JAK2), which is an upstream kinase of STAT3 [8]. Given the most common age of onset and the invariably increase of age during treatment, altered pharmacokinetics, and concomitant polypharmaceuticals may cause a heightened rate of comorbidities [9, 10]. In fact, age-related alterations in hepatic metabolism and decline in glomerular filtration rate markedly reduce drug clearance. The medications most frequently prescribed to RA patients aim to treat painful and inflamed joints. These are corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying anti-rheumatic and immunosuppressant molecules such as the newer TNF-α inhibitors [11, 12]. However, their use is also associated with several drawbacks such as gastrointestinal side effects, immunodeficiency with serious infections, leukocytoclastic vasculitis, or increased cancer risk [13-15]. These limitations have prompted several research streams to search for alternative non-chemical therapeutics [16-18]. There is a wide use of complementary and alternative medicine (CAM) utilized, and half of CAM users with RA do not inform their physicians, either considering these compounds of liberal use to be “natural and safe” or also for a deliberate choice fearing the disapproval of their physicians. However, this attitude, while being of no benefit for the advancement of potential CAM integration, on the other hand, may unsuspectingly expose RA patients to interactions or side effects [19-21]. Indeed, studies have demonstrated associations between CAM use and the increasing number of comorbidities [22). Nonetheless, several clinical studies have been attempted as analyzed in 2011 by Ernst 2011 et al. [23] and in a more recent meta-analysis studying Chinese herbal preparations (24). However, as recently pointed out (25) a vast number of reports from either Asian and Western source the majority of trials showed uneven adherence to clear guidelines, owing to a great heterogeneity and limited information regarding the standardization and content of herbal treatment. While phytocompounds have indeed been largely analyzed and under scrutiny, there are only a few, but promising reports on marine biology-derived compounds in the treatment of RA [26-30]. Very recently, a study using a marine extract in RA has shown a benefit effect on inflammatory pathways [31]. About 10 years ago, we had shown that a specific proprietary marine lipoprotein, fish DNA extracts and peptides-rich compound, LD-1227 (Caviarlieri, Lab-Dom, Switzerland) could significantly inhibit IL-1β-induced proliferation and inflammatory reactions by partly inhibiting the transcription factor NF-κB pathway in human chondrocytes ex-vivo derived from osteo-arthritis patients [32]. Later work has suggested that part of the observed beneficial effects of this novel marine extract could not be solely ascribed to its content of EPA/DHA [33-35]. Given that, this novel marine had previously been shown to possess higher anti-inflammatory properties than EPA/DHA in an experimental setting [36], the present study aimed to test it against a high dose of EPA/DHA in clinically stable RA patients.

MATERIALS AND METHODS

Materials: Patients with a history of RA dating back at least 3 years were recruited while assuring their clinical status has been stable for no less than the past 3 months and falling into the category “low disease/moderate activity” (DAS28 < 3,2/>3.2 but < 5.1). By using SAS 9.2 (SAS Institute, Cary, NC, USA), a random number-assigning algorithm was utilized by an external statistician to set up related sealed envelopes. Participants were randomized into two age-, disease duration-, symptoms score- and clinical characteristics-comparable groups, i.e., the LD1227 or control omega-3 group, for three months.

As exclusion criteria were considered the following: bone deformities, serum glucose ≥6.93 mmol/L, moderate-severe dyslipidemia (total cholesterol >260mg/dl and/or triglycerides >250 mg/dl), a history of

cardio- or cerebro-vascular accidents, weight loss over 3kg in the past 3 months, past or current cancer, inflammatory bowel disease, renal or hepatic disease, pregnancy, substance abuse, consistent alcohol consumption, thromboembolism, use of blood thinning medications or food supplements, hypertension, drugs-requiring diabetes, overt hormonal dysfunction-related secondary obesity and whatever relevant dose fluctuation of medication during the study.

At baseline, body mass index (BMI) and clinical information was gathered from all subjects. Dietary intake was recorded in 3-day food records and weight was measured to the nearest 0.1 kg, with 1 kg subtracted to account for clothing. Patients in the LD-1227 group (n = 20) received oral supplementation (800 mg, one cps t.i.d.) containing the marine extract for 12 weeks whereas the other group received Omega-3 (1000 mg/one cap t.i.d) for 12 weeks. Examining physicians and laboratory personnel were assigned to a group blindly. All patients were asked to suspend any other nutraceutical, vitamin, or vitamin-enriched drinks during the study period and abstain from drug dose fluctuations without notice. A total of three tests were conducted (baseline, at 6- and 12 weeks of treatment). All subjects signed a written informed consent.

Treatment for most was based on 5 mg prednisone twice a day, 0.2 mg/kg MTX per week, and at times added cycles of nonsteroidal anti-inflammatory drugs (NSAIDs) or 200 mg hydroxychloroquine daily. On demand, extra NSAIDs were allowed according to the treating physician's judgment who had to be informed. The trial physician was blind to the specific treatment but aware it was a polyunsaturated fatty acid formulation.

Methods: Eligible patients (LD-1227: 20 patients; age-range 39-65, m/f ratio: 4/16 and Omega-3: 20 pts, age range 33-57, m/f ration: 2/18) were asked to fill out questionnaires to evaluate their RA status. Health Assessment Questionnaire (HAQ) is a questionnaire measuring the outcome of RA subjects (37). This was administered following the Stanford Arthritis, Rheumatism, and Aging Medical Information System. Patients' answers are centered on their ability to dress and take care of their hygiene, standing up from a chair, eating, walking, and other routine daily life activities. For VAS (visual analog scale) each patient was asked to score his/her perceived pain intensity along a 100 mm horizontal line, and this rating is then measured from the left edge (=VAS score) ranging from 0 (no pain) and 10 (worst pain) (38). Disease Activity Score (DAS) 28 assesses the degree of disease activity in RA by pooling together: tender and swollen joints biochemical values (39). These three questionnaires were provided at the entry, 6 and 12 weeks afterwards along the treatment.

Endpoints. The primary efficacy endpoint was the improvement in ACR20 and ACR50 (40) and inflammatory cytokine levels. Secondary endpoints were rheumatoid factor (RF) levels, highly sensitive C-reactive protein (hsCRP) levels, cytokines gene expression, pain marker, and erythrocyte sedimentation rate (ESR).

Blood tests. After an overnight fast, individuals were asked to rest in an isolated and quiet room for 5min. Then, vital parameters were assessed, and blood was drawn from the antecubital vein and split in duplicate. A total of 10mls of venous blood was drawn from an antecubital vein and put into an ethylenediaminetetraacetic acid (EDTA)-K_2-coated vacutainer tube. Glucose, cholesterol, HDL-C, triglycerides and hsCRP were assessed using commercial kits (Synchron LXT 20 analyzer, Beckman-Coulter, UK) with all assays used a Biochip array to quantitatively detect multiple analytes from each single subject. The kit was each time subjected to appropriate calibrators and quality controls.

Serum Cytokine assay. From separate tube. the serum was immediately centrifuged at 2000 *g* for 15min at 4°C and within 30 minutes 300µl aliquots of the supernatant were liquid nitrogen-frozen and stocked at -

80°C until analysis. Cytokines were assayed by the Bio-Plex Pro human cytokine Th1/Th2 immunoassay 96-well kit (Bio Rad, Germany), containing related antibodies. This multiplex kit assays MCP-1, IL-1β, IL-6, TNF-α, and interferon-gamma inducible protein-10 (IP-10) using Human Ultrasensitive Cytokine 10-Plex Panel (Life Technologies, Carlsbad, CA, USA). Assays were read by a Luminex 200 Analyser (Luminex Corporation, Austin, USA). The serum soluble IL-15Rα level was assessed using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) (CUSABIO, USA). The intra- and inter-assay coefficients of variation were < 10%. Measurement sensitivity is shown in table 3. All samples were tested in duplicate and the average of the 2 values was used for the final data analysis. The intra- and inter-assay coefficient of variation of < 9% was taken as a pre-requisite to test the samples before intervention and 3 months afterwards.

Table 1. Characteristics of the participants

Parameter	Omega-3 (20pts)	LD-1227 (20 pts)	p value
Age (years)	53.2±6.3	48.7 ± 5.6	ns
BMI	25.4 ± 2.1	24.7 ± 2.6	ns
Duration of disease (months)	48.4 ± 2.1	41.7 ± 6.6	ns
RF positivity	100% positive	100% positive	ns
ESR (mm/h, mean ± SD)	38 ± 9.3	37 ± 10.4	ns
CRP (mg/dl, mean ± SD)	9.1 ± 4.3	10.2 ± 6.4	ns
Morning stiffness (min. ± SD)	91 ± 9	94 ± 10	ns
Tender points (mean ± SD)	8 ± 3	9±2	ns
Swollen joints, (mean ± SD)	4 ± 2	3 ± 1	ns
Pain (VAS, 0–10), (mean ± SD)	5.7 ± 1.2	4.8 ± 1.3	ns
HAQ score (mean ± SD)	0.8 ± 0.3	0.8 ± 0.2	ns
DAS28	4.0 ± 1.4	4.3 ± 0.9	ns
Extra NSAIDs, number/month	11 ± 3	9 ± 2	ns

Abbreviations: hsCRP = highly sensitive C-reactive protein; ESR = erythrocyte sedimentation rate; HAQ = Health Assessment Questionnaire; VAS = visual analogue scale; DAS = Disease Activity Score; ns: not significant.

Table 2. Cytokine assay sensitivity (measured by Bio-Plex Pro human cytokine Th1/Th2 immunoassay)

Biomarker	Calibration range (pg/mL)	Sensitivity (pg/mL)
IL-6	0-800	1.0
TNF-α	0-1500	3.5
IL-1β	0-600	1.9
CXCL1	0-500	0.5
IL15R-α	0-2500	7.8
IP-10	0-2500	2.5
MCP-1	0-1500	11.6

Serum SP concentration was assayed using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (R& systems, Minneapolis, MN, USA). The ELISA plate reader (Stat-2100, Awareness Tech, Inc., USA) was set at 450 nm absorbance. The detection limit was 25 pg/mL and Intra- and inter-assay coefficients of variation were 9% and 12%, respectively.

Safety was monitored throughout the study. The frequency and severity of adverse effects or adverse treatment reactions were scrutinized at each visit.

Clinical and standard hematological and biochemical tests and urine analysis were also monitored.

Cytokines gene expression. PBMC was isolated from whole venous blood collected into heparinized tubes and diluted with an equal volume of PBS. Cell separation was carried out by density gradient centrifugation. For each sample, two l5-ml centrifuge tubes were used to layer 7 ml of diluted blood onto an equal volume of Ficoll-Hypaque. After centrifugation (30 min/450g at 20°C), the mononuclear cell layer was pipetting-removed, washed twice with PBS, and centrifuged (10 min/275g at 10°C) to be finally stored at −80◦. Total RNA was extracted following the manufacturer of the RNeasy kit (Qiagen, Crawley, West Sussex, UK). Two micrograms of total RNA were used for cDNA synthesis and later gene expression assessment in Real-Time PCR.

Gene expression analysis. The concentration and purity of the recovered RNA were tested by ultraviolet absorbance at 260 and 280 nm and its integrity by agarose gel electrophoresis. TaqMan Gene expression assays (ABI Prism 7900 HT, Applied Biosystems) were used to quantify gene expression. The expression of cytokine mRNA for each sample was then expressed as an arbitrary ratio over β-actin. The sequence of primers was designed for an annealing temperature of 60°C using Primer 3 software except from β-actin primers (a housekeeping gene) which also anneals at 60°C. The primers used for relative quantification of targeted gene expression are as follows. For TNF-a: forward primer, 5'-GCCACCACGCTCTTCTGT-3'; reverse primer, 5'-·GGCTACGGGCTTGTCACTC-3'; for IL-6: forward primer, 5'- GTATGAACAGCGATGATGCAC-3'; reverse primer, 5'-GAAACGGAACTCCAGAAGACC-3'; For MCP-1: forward primer, 5’-TTCTCAAACTGAAGCTCGC-3’; reverse primer, 5'-AAGCTAGGGGAAAATAAGTT; for IL-1β: forward primer GACCTGTTCTTTGAGGCTGAC and reverse primer TTCATCTCGAAGCCTGCAGTG; For β-actin: forward primer: 5'-TCCCTGGAGAAGAGCTACGA-3'. Reactions were normalized to a final volume of 25 µl, IX reaction buffer, 0.2 mM of each deoxynucleotide, 2.5 mM MgCl2 0.15ItM of each primer, 1:100.000 SYBR Green I (Molecular Probes, Leiden, Netherlands) and 0.4 U of HotStart DNA Taq Polymerase. For each target primer set, a validation experiment was assessed to prove the PCR efficiency and in accordance with the reference gene. Each sample was assayed in duplicate, and each target gene was normalized to the reference gene expression. Agarose gel electrophoresis was performed to confirm that there were no primer dimers together with single-product amplifications.

Statistical Analysis: Patient baseline characteristics and their parameters 3 months after treatment, including CRP, ESR, cytokines, HAQ, VAS, and DAS28 were compared using chi-squared tests for categorical variables and Fisher's exact test for continuous variables. Statistical significance was defined as $P \leq 0.05$. All statistical analysis was performed using SPSS, version 16.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

No significant change occurred in the routine blood chemistry whatever the treatment (data not shown). Overall, the symptoms reported by the patients after treatments were significantly relieved. There were significant differences in the symptoms of RA between the LD-1227 groups and the omega-3 group.

As shown in table 3, at the end of the study, mean morning stiffness showed a not significant trend decrease in the omega-3 group from 91 ± 9 min. to 77±12 min. ($p<0.65$) whereas this improvement was more relevant in the LD-1227-treated group (94±10 min vs 39±21min, $p<0.01$). The average number of the tender joints and swollen joints was reduced by both treatments but, as for tender points variable, LD-1227 was significantly more effective than omega-3 ($p<0.05$). The same applied to the VAS pain score. Both treatments comparably improved the HAQ score while only LD-1227 beneficially affected the DAS28 score ($p<0.05$). Both treatments reduced the need for extra NSAIDs ($p<0.05$), but this number was statistically lower in the LD-1227-treated group ($p<0.05$).

Table 3. Clinical characteristics of RA patients in the two treatment groups

Parameter	LD-1227	Omega-3	p value, LD-1227 vs Omega-3
BMI	24.8 ± 2.4	25.2 ± 3.3	ns
RF positivity	100% positive	100% positive	ns
ESR (mm/h, mean ± SD)	15 ± 3.2*	17 ± 4.6*	ns
CRP (mg/dl, mean ± SD)	5.01 ± 1.17	8.29 ±1.31	p<0.01
Morning stiffness (min. ± SD)	39 ± 21*	77 ± 12	p<0.01
Tender points n. (mean ± SD)	4 ± 2*	6 ± 2*	p<0.05
Swollen joints n. (mean ± SD)	2.6 ± 1.6*	3.2 ± 3.3*	ns
Pain (VAS,0–10), (mean ± SD)	2.1 ± 1.2*	3.9 ± 1.1*	p<0.05
HAQ score (mean ± SD)	0.3 ± 0.2*	0.4 ± 0.3*	ns
DAS28	2.2 ± 1.2	3.5 ± 0.9	p<0.05
Extra NSAIDs need, n./month	4 ± 2	9 ± 2	p<0.05

Abbreviations: CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; HAQ = Health Assessment Questionnaire; VAS = visual analogue scale; DAS28 = Disease Activity Score. *p<0.05 vs baseline.

ACR rate improvement at 12 weeks. Fig.1 shows the end of the study ACR scale modification following the two treatments employed. As compared to baseline, both treatments brought about a significant benefit but the one provided by LD-1227 was significantly more consistent as compared to omega-3 at either ACR 20% or ACR 50% scale evaluation (p<0.01). Under ACR 70% scale assessment, the two treatments seemed to be comparable albeit at a poor response and with a trend better performance of LD-1227 but the limited number of cases did not allow a proper evaluation.

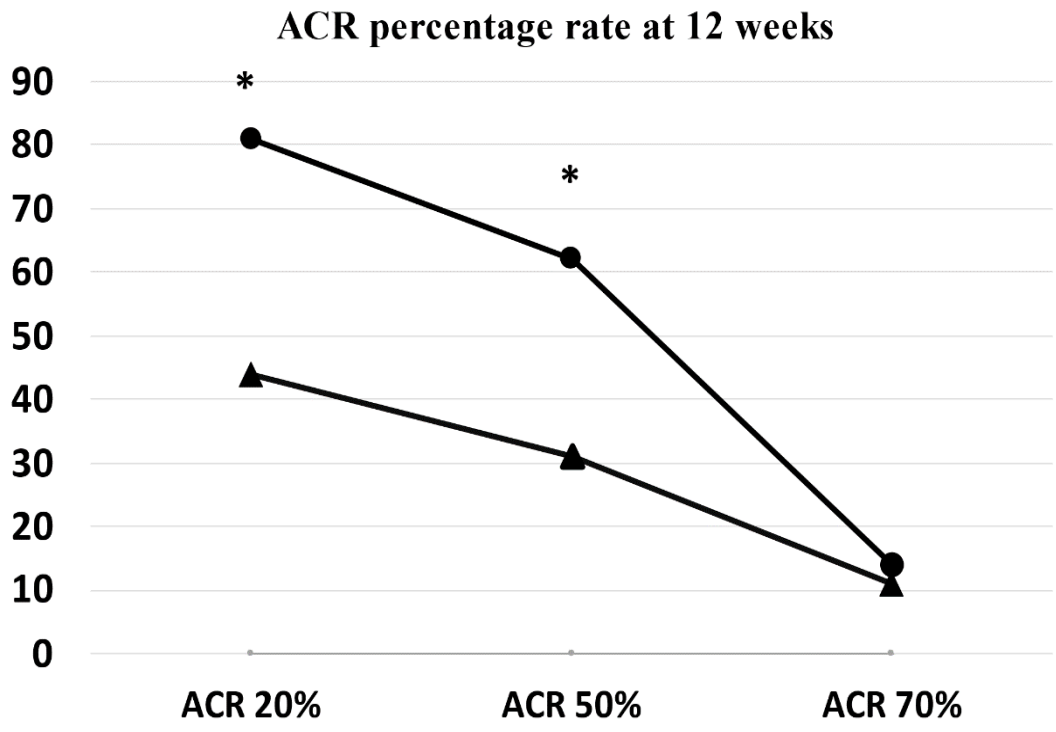


Figure 1. ACR percentage of quantitative pain decrease at the 3rd month observation. Round dots: LD-1227, triangles: Omega-3. *p<0.05 vs Omega-3.

Time-course modification of pain marker. Fig. 2 shows the time course variation of SP plasma level. Omega-3 did not affect this parameter, although at the 3-month observation a non-significant trend was noted. On the other hand, LD-1227 at 6-weeks showed a significant decline ($p<0.05$ vs baseline) which reached a higher significance at 12 weeks ($p<0.01$ vs baseline, $p<0.05$ vs 6 weeks level)

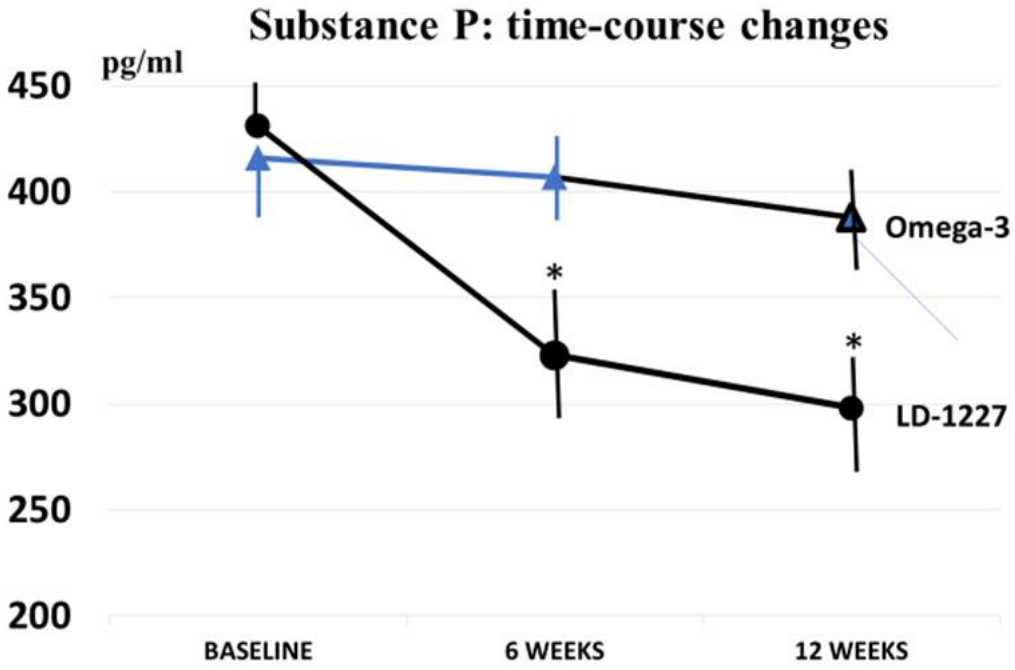


Figure.2. Serum SP concentration assayed using an enzyme-linked immunosorbent assay. *$p<0.01$ vs baseline and omega-3 values.

Cytokine assessment. All cytokine plus CXCL1 tested in RA subjects showed a significant increase when compared to the control (table 4). Serum IL-6 levels were correlated with clinical parameters at baseline, including HAQ, DAS28, CRP and ESR; this did not apply when considering TNFα levels. Indeed, omega-3 treatment was ineffective in all other biochemical parameters assessed, unlike what observed with LD-1227 which enabled a significant decrease. In LD-1227 group it also appeared a direct correlation between the chemokine CXCL1 with TNF-α and IL-1β before and after treatment ($r: 0.87$, $P < 0.01$). Interestingly, omega-3 was ineffective in reducing the level of IL-1β and of CXCL1. At baseline, serum MCP-1 levels in both groups were significantly correlated with the combined DAS28-CRP ($r: 0.78$, $p<0.05$) such correlation was maintained also at post-treatment check in LD-1227 group ($r:0.68$, $p<0.05$). The wide scattering of data of post-treatment values of IL-15R-α in the omega-3 group did not allow to yield a statistical significance, despite a trend decrease, unlike what observed in LD-1227 group ($p<0.01$ vs baseline and $p<0.05$ vs omega-3 group value). IP-10 was significantly correlated to CRP in the full set of patients at entry ($p<0.05$). However, at post-treatment check, this chemokine was significantly associated to pain and DAS28 only in LD-1227 group ($p<0.05$).

Table 4. Cytokine profiles in the two treatment groups

Biomarker	LD-1227	Omega-3	Statistics
IL-6 (pg/mL) At entry End of study Control 2.3±0.6	22.2±4.9* 14.8±5.9§	20.7±2.5* 13.7±5.4§	ns ns
TNF-α (pg/ml) At entry End of study Control 14.4±5.3	24.5±7.1* 17.2±6.2**	25.2±5.4* 20.3±5.7	ns < 0.01
IL-1β (pg/mL) At entry End of study Control 2.2±0.3	26.6±4.3* 12.2±4.2**	24.9±5.3* 19.6±9.6	ns < 0.01
MCP-1 (pg/ml) At entry End of study Control 158.4±9.7	222.7±29.3* 193.6±9.8§	231.2±31.6* 199.8±17.7§	ns ns
CXCL1 (ng/mL) At entry End of study Control 2.0±0.3	2.7±0.2 2.1±0.3*	2.8±0.4 2.4±0.6	ns p<0.05
IL-15R-α (pg/ml) At entry End of study Control 322.3±60.5	1214.7±282.6* 488,6±147	1146±198.5* 974.1±238.4	ns p<0.05
IP-10 (pg/mL) At entry End of study Control 112.0±89.3	482.7±150.5* 246.5±132.3*	489.8±166.4* 398.7±116.6	ns p<0.05

Serum cytokine level at entry and after 3 months supplementation with either LD-1227 or omega-3. *p<0.05 vs baseline. Statistic between the two treatment groups is shown in the right column.

Gene expression studies. At baseline. all inflammatory markers gene expressions showed a significant upregulation as compared to healthy control which represented the arbitrary unit normalised to gate keeper gene equal to 1 (p<0.01, fig.3). Post-treatment gene expression check showed that, no matter the treatment, IL6. TNF-α and MCP-I were significantly downregulated. None of them reached the healthy control value, though. Moreover, the downregulation of MCP-1 yielded by LD-1227 was significantly more pronounced as compared to omega-3 group (p<0.05). LD-1227 was the only treatment which yielded a partial but significant downregulation of IL-1β and CXCL1 (p<0.05 vs baseline).

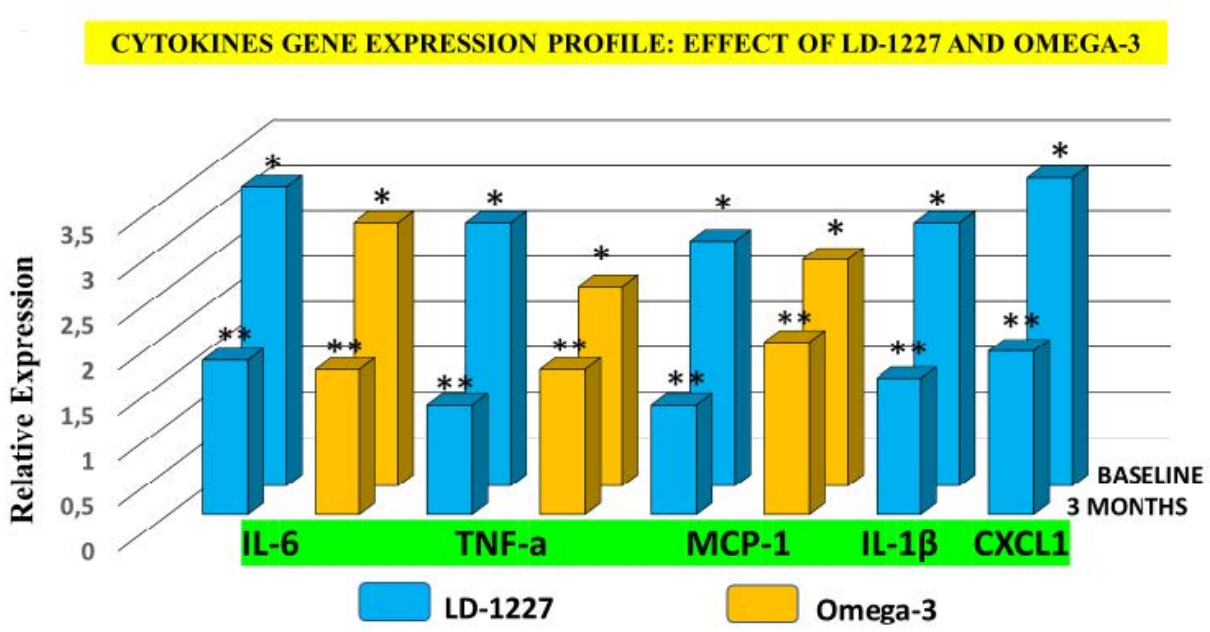


Fig.3. Gene expression of cytokine which showed altered level in the serum. *p<0.01 vs baseline; **p<0.05 vs entry level. LD-1227 and omega-3 comparably downregulated IL-6, TNF-alpha and MCP-1. Only LD-1227 downregulated IL-beta and CXCL1.

DISCUSSION

RA represents a multifaceted disease associated to several comorbidities and with an 85% higher mortality risk compared to the general population. Thus, RA is understandably bearing a significant negative effect on to quality of life.

Several medications endowed by anti-inflammatory and immunomodulatory activity are currently used in medical practice but there are also burdened by relevant side effects, thus imposing great caution in their medium-long term utilization. The long list of these drugs includes either drugs over the counter (acetaminophen, aspirin, ibuprofen, or naproxen) or requiring specialist's prescription (methotrexate, gold salts, penicillamine, sulfasalazine, hydroxychloroquine and related combinations of them, besides more recent biological response modifiers). This has raised also the interested towards the search of natural anti-inflammatory compounds [39-48]. However, specific data in RA have remained scattered and often without a sound-controlled protocol design. In general, vegan diets rich in antioxidants and lower in saturated fats such as also reported in the Mediterranean diet have demonstrated some benefits in regard to pain, but not other aspects of disease activity. In the present study as a therapeutic control group, we chose omega-3 that is one of the most common nutraceuticals used by RA subjects and endowed by some supporting literature [49-52]. These omega 3-centered studies have some limitation for the lack of a control arm. Moreover a 5-year study has shown that omega 3 fatty acid supplementation reduced the autoimmune disease rate by a not statistically significant 15% rate [53]. From the present study, it suggests that LD-1227 may reduce the level of CXCL1 by inhibiting the expression of TNF-α and IL-1β, thus inhibiting immune cell recruitment and proinflammatory effects. Moreover, from the reduced level of CXCL1 one can infer that a certain degree of inhibition of peripheral neurons excitatory mechanisms and related hyperalgesia also play a role. Except for IL-6 and TNF-α where both treatments showed comparable curbing effect, LD-1227 showed a significantly suppression of the other cytokines higher

than omega-3. A note could be raised regarding the dose of Omega-3, however large doses of at least 2.7g EPA + DHA, while being more hypothetically liable to achieve potentially better outcomes in RA, have raised, although conflicting, concerns on their risk of bleeding [54], besides bearing lower compliance for taking several pearls a day. On the other hand, the link between n-3 PUFA and CRP, for example, is unclear since a 12-week supplementation doses of n-3 FA up to 6 g/day, did not decrease CRP concentrations when compared to the placebo group [55]. When pooling the analytical biochemical and non-parametrical factors such as, cytokines, chemokines, SP and CRP level with DAS28, HAQ, pain assessment and extra need of NSAIDs, one can conclude that LD-1227 may play a significant role on the management of RA and with a spectrum and mechanisms of actions distinct from the canonical omega-3. Finally, the safety of this intervention has also been conformed in other published research (32-36) and this hold particularly true considering the recent warning about at times risky efficacy-toxicity balance in treating RA [56]. We could not assess JAK-2, STAT gene status, and possible variants; this deserves more investigation in the future as well as a larger and cross-over design approach. Finally, future far larger studies using different non-chemical approaches may be engaged in view to prove higher effectiveness of some or benefit from combination schemes.

Conclusion: One can conclude that LD-1227 has the potential to play a significant role on the management of RA as adjuvant treatment on top of guidelines therapy and likely to decrease its pharmacological burden. It appeared to display a specrum and mechanisms of actions to be expanded and, not last, distinct from the canonical omega-3 while being devoid of any side effect or tolerability issues.

Abbreviations: Rheumatoid arthritis (RA), Health Assessment Questionnaire (HAQ), erythrocyte sedimentation rate (ESR), visual analogue scale (VAS), Disease Activity Score (DAS) 28, American College of Rheumatology (ACR20), nonsteroidal anti-inflammatory drugs (NSAIDs), macrophage chemoattractant protein-1 (MCP-1), interleukin 1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor- α (TNF-α), interferon-gamma inducible protein-10 (IP-10), interleukin 15R-α (IL-15R-α), Chemokine Ligand 1 (CXCL1).

Authors' contribution: FM, AL and NZ designed the study and followed it up; RR, FH and US contributed to the discussion; SR, NA and CA contributed to data analysis.
All authors declare no competing interest with the present study.

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