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A randomized, controlled trial on the effectiveness of a proprietary marine lipo-peptide formula vs omega-3 on cytokines profile, anxiety, and pain symptoms in patients with fibromyalgia

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ABSTRACT

Objective: The aim of the present study in an RCT manner (physicians and patients) a novel lipo-peptide marine compound, LD-1227, on physical-, emotional- and functional-related symptomatic complaints in fibromyalgia patients as well as inflammatory cytokines profile and gene expression while using omega-3 as a control group.

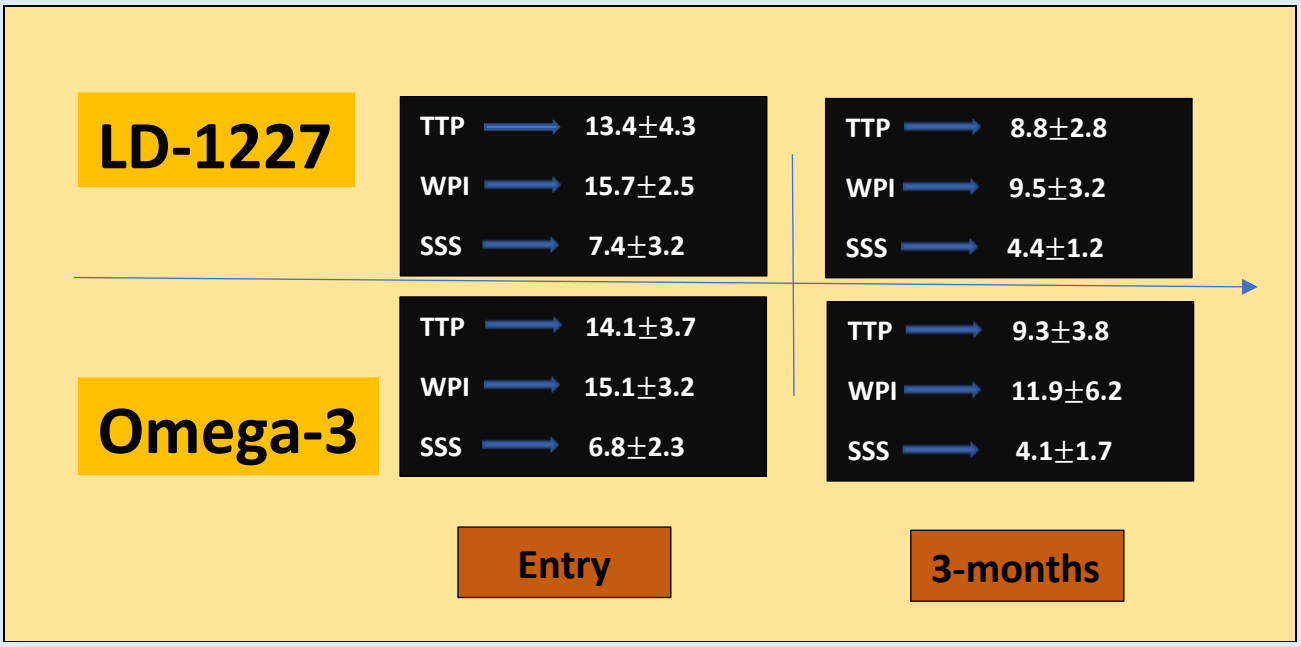
Methods: The following questionnaire-based or clinical evaluation-based parameters were evaluated: widespread pain index [WPI] patient global impression of change, total tender points [TTP], fibromyalgia impact questionnaire, Beck depression inventory, fatigue severity ratings, cognitive symptom severity, symptom severity score [SSS] and weekly pain intensity ratings. Additional biochemical and gene expression analysis of cytokines (IL6, TNF-α, IL-1β, MCP-1, IL-8, IL-13, IL-1α, and GM-CSF) was performed as well. Data were analyzed with either a paired t-test or the Wilcoxon signed rank test depending on the parametric or non-parametric distribution.

Results: Comparing the data from before and after treatment for Group B indicated a statistically significant reduction (p=0.05) in TTP, WPI score, and SSS score. These data suggest a positive effect of a 3-month treatment with the LD-1227 but not omega-3 treatment on Fibromyalgia pain and related anxiety/depressive symptoms. Inspections of HRV and

Cytokines found a statistically significant improvement after LD1227 treatment. Unlike the group supplemented with omega-3, the treatment with LD-1227 brought about a decrease in WPI and weekly pain intensity symptoms for the majority of participants. The pre-and post-treatment data for Group B indicated a statistically significant reduction (p=0.05) in TPC, WPI, and SSS scores. No adverse events were reported.

Conclusion: These results provide the first indications that the LD-1227 treatment has a statistically significant effect on the recognized fibromyalgia diagnosis metrics of WPI, TTP, and SSS as well on inflammatory markers and parasympathetic balance.

Keywords: fibromyalgia, marine compound, lipopeptide, cytokines, anxiety, pain, LD-1227.



INTRODUCTION

Fibromyalgia (FM) is afflicting between 2 and 7% of the population in most nations, with a female to male ratio of about 9:1, bearing the high social cost and work inability [1]. However, it still poses a challenging task whenever a comprehensive pathophysiological understanding is approached. This is due to the proteiform variety of symptomatology characterized by chronic diffuse Musculoskeletal pain with increased sensitivity to pressure together with different degrees of fatigue, depressive mood, memory and concentration difficulties, sleep disorders, migraine, irritable bladder, and gastrointestinal discomfort [2-5]. The categorization of the pathophysiological mechanisms behind FM is still interfered with and blurred by psycho-emotional variables [6] which, by definition, are hard to objectivize. The matter is further complicated by the placebo effect of several non-invasive body treatments as shown in one study where both the control group and FM participants who received magnetic stimulation, reported similar improvement in Clinical Global Impression scores [7]. Nonetheless, there is a body of evidence about a

biochemical milieu characterized by a higher expression of pro-inflammatory cytokines. However, a tentative anti-inflammatory probiotic intervention has shown to be ineffective on related gastrointestinal symptoms in a well-built study [8] against others which, less convincingly, were limited to report a subjective improvement [9]. Similarly, despite theoretical assumptions on the benefit of oxygen-ozone therapy [10], inconsistent results often devoid of sound proven biochemical and molecular biology data still put its hoped benefits in the hazy realm of anecdotes [11].

Over 10 years ago and after the 2016 revision, the American College of Rheumatology pointed out the key diagnostic criteria for FM, i.e. Widespread Pain Index [WPI] and Symptoms Severity [SS] scale together with the presence of at least 11 out of 18 tender points on examination and chronic widespread musculoskeletal pain [CWP] in the axial skeleton in at least two contralateral body quadrants and lasting for at least three months [12].

Given the elusive boundaries of FM pathomechanisms and the co-existence of psychological interfering overlaps, several drugs have also been tested and FDA has approved drugs of rather cautious use such as duloxetine, pregabalin, and milnacipran [13, 14]. However, such chemicals are not devoid of risks [15, 16] and their benefit/toxicity ratio is at times a bit thin, making the fundamental of medicine “primum non nocere.” (First do no harm) an issue. In this respect, several nutritional and nutraceutical interventions have been suggested and reported in albeit small and short-duration trials [17-24], including also omega-3 [25-28].

Within the growing research on marine biology, in 2012 Catanzaro et al. had shown that a specific DNA peptides-rich marine extract (LD-1227) could significantly inhibit IL-1β-induced inflammatory reactions by curbing NF-κB transcription factor in ex-vivo chondrocytes derived from osteoarthritis patients [28]. Later work from our group further strengthened the suggestion that moieties other than EPA/DHA had to be advocated for their biological action as we proved that it is able to significantly protect the brain structure redox system to a higher degree than DHA [29]. Moreover, from in vitro study, it appears that marine bioactive compounds, through its wide array of small unsaturated fatty acids, phospholipids, and neurotransmitter precursors. This was confirmed two years later in an experimental spontaneous hypertensive model where, unlike the EPA/DHA-group, LD-1227-treated rats displayed a significant reduction in endothelial alteration with severe subcellular injury [30]. Although the intimate mechanisms of actions of LD-1227 are still under study, it seems that it has a multifaceted anti-inflammatory action. The aim of the present study was to test LD-1227 on typical symptomatic parameters observed in fibromyalgia (FM) patients together with potential modifications in their reported abnormal inflammatory profile. In this RCT study, omega-3 was chosen as a control group, being the most over-the-counter food supplement used by these patients and for possibly misleading similarities with the tested marine lipo-peptide compound.

MATERIALS

Fifty-four FM patients (educational level: from high school diploma upward) were initially recruited after a preliminary screening in Northern Italy. The following exclusion criteria were adopted: unexplained weight loss >3kg in the past 3 months, prior or ongoing cancer, impending or overt diabetes, renal, liver, or hematological disease, severe lipid disorders requiring statins, a history of cerebro- or cardio-vascular accidents, long-standing and severe digestive functional disorder (as from Rome III classification), consistent alcohol consumption, severe or unstable hypertension or overt hormonal dysfunction-related secondary obesity.

Participants who reported mouth dryness which could bias saliva sampling and diurnal cortisol assessment were also excluded. Inclusion criteria were a clinical diagnosis of FM (see below), no active physical exercise, or use of any supplements or medical medications.

METHODS

Each selected subject provided written informed consent and HIPAA approval together with the following demographic and clinical information while the Declaration of Helsinki were duly applied. All procedures were approved by an independent Ethical Committee for nonpharmacological research (ReGenera Research Group for Aging Intervention, trial n. 127/2022). This was a parallel-group two-arm cluster randomized trial with no control group carried out in a free-living setting. Patients were asked to strictly stick to the study intervention over the course of the trial and to immediately report any changes in their scheduled therapy. However, those who halted, or modified pain medications were excluded or kept in a 3 week wash-out period prior to recruitment.

Forty-three female participants, whose ages ranged from 29 to 66 years (mean age: 44 years), that was diagnosed with FM according to the 2010/2016 ACR diagnostic criteria were finally enrolled in the study which was conducted from February 2022 to June 2022. Two participants belonging to the Omega-3 group withdrew from the study for personal reasons and their partial post-therapy data were not obtained. Patients were sequentially numbered a randomized into two groups, (treating physicians and patients being blinded for the therapy). Group A patients (n=21) were supplemented with LD-1227, 1c twice a day and Group B (n=20) were given Omega-3, 900mg 1c twice a day. Given the preliminary nature of the study, the relevance of symptomatic benefit to achieve, and limited access to FM patients, it was set a “time to event” at 3 months.

Primary Outcome measures were as follows: Pain by using the Visual Analogue Scale (VAS) with scores ranging from 0 (no reported pain) to 10 (most severely perceived pain). Repeated in-house tests aimed to assess the internal robustness yielded a reliable 0.68-0.81 consistency value. Fibromyalgia Impact Questionnaire [FIQ] was applied to evaluate the overall health status with FM-related functional disability asking the patient to score the severity of perceived pain over the past week by a Likert-like scale [31] over 21 questions. In particular, at the entry into the study, all subjects answered questions regarding the extent of body pain Widespread Pain Index, [WPI]; range 0–19), and the number and severity of the centralized symptoms of chronic pain (Symptom Severity Scale [SSS] (32).

The 40-item-powered State-Trait Anxiety Inventory (STAI) [33] was applied to evaluate the anxiety level by using a Likert-like scale (from 0 intensity and frequency to 4, i.e. very intense and frequent). A similar quali-quantitative self-reported assessment was used to employ the Beck Depression Inventory (BDI) [34] with each item ranging from 0 to 3, with a cumulative score ranging from 0 to 63).

Each participant also underwent trained osteopath palpation on the 18 specific anatomical tender points as defined by the 1990 ACR FM classification. For each point, patients rated the level of tenderness (from 0 to 3, for none, mild, moderate, and severe, respectively). The total summing up tender points [TTP] is ranging from 0 to 54. After completing the entry clinical assessment and questionnaires, patients were instructed to collect saliva samples at home for diurnal cortisol measurement on the day preceding scheduled visits (at entry, 6 weeks, and 12 weeks afterward). Each time, the sample was collected within 2h to be brought to the laboratory or personally delivered by the patient.

Secondary outcomes: biochemical and heart rate variation (HRV) assessments:

Blood testing procedure: After overnight fasting, a total of 10ml of venous blood was withdrawn and in each case with minimum stasis. One part was put into dextrose K3 EDTA anticoagulant citrate dextrose solution added with 0.8% citric acid, and 2.2% trisodium citrate. Routine blood tests (full blood count, liver and lipid profile, renal function) including hsCRP were assessed by commercial kits (Synchron LXT20 analyzer, Beckman-Coulter, UK). Biochip array technology performing simultaneous detection of multiple specific cytokines and growth factors was applied.

Cytokines plasma level and gene expression: Cytokines most commonly reported as abnormal in FM were tested, From separate tube, the serum was immediately centrifuged at 2000g/15min at 4°C and 300µl aliquots of the supernatant, transferred into Eppendorf tubes, were frozen in liquid nitrogen and quickly stocked at -80°C until analysis. Cytokines were assessed by means of the BioPlex Pro human cytokine Th1/Th2 immunoassay 96-well kit (Bio-Rad, Germany), endowed with magnetic beads and related antibodies. This multiplex kit assays IL-6. IL-8, IL-18, IL-1β, IL1α, GM-CSF, MCP-1, IFNγ, and TNF-α by using a Luminex 200 Analyser (Luminex Corporation, Austin, TX, USA. All samples were calculated from the average of duplicate analyses with an intra- and inter-assay coefficient of variation <8% whereas those with undetectable values were assigned by default the lower detection limit of the assay. Tests were done before the intervention, 1 month, and 3 months afterward.

Cytokines gene expression was carried out only for cytokines whose serum levels were found to be significantly different from age-comparable controls (twenty gender/age-comparable healthy staff). PBMC were isolated from whole venous blood collected into heparinated tubes and diluted with an equal volume of PBS. Cells stratification was obtained by Ficoll-Paque density gradient centrifugation. For each sample, two centrifuge tubes were used to layer 7 ml of diluted blood onto an equal volume of Ficoll-Paque. The suspension was centrifuged at 450*g*/30min and 20°C. The mononuclear cell layer was manually pipetted out, washed twice with PBS, and centrifuged at 275g/10min for at 10°C following each wash and stored at −80°C. Total RNA was extracted using the Gold RNA PCR Core Kit (PE Biosystems), according to the manufacturer's instructions. Two micrograms of total RNA were utilized for cDNA synthesis and Real-Time PCR gene expression analysis.

The concentration and purity of the recovered RNA products were checked each time by ultraviolet absorbance at 260-280 nm and with agarose gel electrophoresis to test their integrity. TaqMan Gene expression assay (ABI Prism 7900 HT, Applied Biosystems) was used to quantify gene expression. The expression of cytokine mRNA for each sample was conventionally expressed as a ratio of the mRNA quantity to that of β-actin as a housekeeping gene. The sequence of primers is set at an annealing temperature of 60°C using Primer 3 software. The primers were as follows. For TNF-a: forward primer, 5'-GCCACCACGCTCTTCTGT-3'; reverse primer, 5'-·GGCTACGGGCTTGTCACTC-3'. For MCP-1: forward primer, 5'-TTCTCAAACTGAAGCTCGC-3'; reverse primer, 5'-AAGCTAGGGGAAAATAAGTT; for IL-6: forward primer, 5'- GTATGAACAGCGATGATGCAC-3'; reverse primer, 5'- GAAACGGAACTCCAGAAGACC-3; for IL-8: 5'-

CATACGAATTCCATGGGCAAGCTTGAATCTAAATTA-3' reverse primer: 5'-CATATGGATCCGCTAGTCTTCGTTTTGAACAG; for IL-1β: forward primer GACCTGTTCTTTGAGGCT GAC and reverse primer TTCATCTCGAAGCCTGCAGTG; for β-actin: forward primer: 5'-TCCCTGGAGAAGAGCTACGA-3'; reverse primer, 5' – ATCTGCTGGAAGGTGGACAG-3'; for IL-13 forward primer,5'-GAG TGT GTT TGT CAC CGT TG-3' and 5'-TAC TCG TTG GTC GAG AGC TG-3' for the downstream primer. Reactions were normalized to a final volume of 25 µl, 2.5 mM MgCl2, 0.2 mM of each deoxynucleotide, 0.15ItM of each primer, IX reaction buffer, and 1:100,000 SYBR Green I (Molecular Probes, Leiden, Netherlands) and 0.4 U of HotStart DNA Taq Polimerase. For each target primer set, PCR efficiencies were validated beforehand as well as the reference gene within a range of 90–100%. Agarose gel electrophoresis was employed to confirm each time the presence of single-product amplifications each.

Salivary cortisol level measurement: All patients were instructed to follow the protocol to collect their saliva in sample tubes at home at 30 min after awakening and standing up to start the day (between 7.00 and 8.00am), this being previously tested in-house as the most responsive and representative and corroborated by literature reports [35]. Patients were asked not to brush their teeth, gargle, eat or drink for at least 1h before sampling. All samples were taken to the lab the next day and kept at −20 °C until later analysis using commercial Elisa assay kits, Assay Max ELISA Kits (Assaypro, St. Charles, MO, USA). The cortisol at awakening was derived as the difference between levels at the awakening time and 30 min afterward.

Heart Rate Variability (HRV). We used Body Health Analyzer Professional Edition (Binacor, Poulsbo, Washington, USA). The system consists of a Bluetooth finger device connected to a dedicated software analysing heart rhythm variation and calculating a number of related sympathetic/parasympathetic algorithms. The tests were always performed on patients put a rest for 10min on a comfortable bed, in a silent, slightly warmed room. A visual analysis of raw data for possible movement artifacts and ectopic beats or breaths was carried out in each study.

Statistical analysis. Because of the limited sample size, Mann–Whitney U tests and chi-square tests were used to study intergroup differences in clinical profiles, subjective cognitive complaints, and diurnal cortisol levels. Correlation analysis was used to probe the association among demographic, clinical profiles, and biochemical analyses by using SPSS version 22 (IBM Inc., Armonik, NY, USA). A critical alpha level of 0.05 was used for all analyses.

RESULTS

No adverse effect connected to the treatment employed was reported by participants and no significant change occurred in the routine blood chemistry (data not shown). Table 1 summarizes the results obtained by analysing the multiple questionnaires. It appeared that both treatments comparably improved either TTP or SSS analysis. However, FIQR, WPI, and STAI showed a significantly better improvement in the group supplemented with LD-1227 ($p<0.05$). Moreover, unlike LD-1227 ($p<0.05$), Omega-3 treatment did not affect BDI-II depression evaluation, and the significant benefit exerted in STAI-I ($p<0.05$) was still less robust than with LD-1227.

Table 1. Modification of FM clinical parameters after 3 months of treatment with LD-1227 or Omega-3

	LD-1227 21 patients		OMEGA-3 20 patients		
	entry	3 months	entry	3 months	**p value LD-1227 vs omega-3**
BMI	21.8±2.7	22.4±3.5	22.2±4.4	23.1±2.5	**ns**
FIQR	61.2±9.8	23.4 ±7.2*	59.7 ± 11.9	33.3 ± 9.9*	**p<0.05**
WPI	15.7±2.5	9.5±3.2*	15.1±3.2	11.9±6.2	**p<0.05**
TTP	13.4±4.3	8.8±2.8 *	14.1±3.7	9.3±3.8 *	**ns**
SSS	7.4±3.2	4.4±1.2*	6.8±2.3	4.1±1.7*	**ns**
BDI-II	24.4±10.2	12.8±8.2*	25.7±9.5	20.5±10.3	**p<0.05**
STAI score	21.2 ± 8.3	10.3 ± 6.8*	23.1± 4.6	16.1± 7.8*	**p<0.05**

Cytokines and hsCRP plasma level. As compared to healthy control, patients with FM showed a significantly elevated level of IL6, TNF-α, IL-1β, MCP-1, IL-8, and hsCRP and lower IL-13 (table 2). The other parameters were within normal limits. Moreover, BMI was positively correlated with IL-6 and MCP-1 ($p < 0.05$) in the whole population at the entry, whereas age and BMI were not correlated to other markers. Post-supplementation, cytokine levels did not significantly correlate with anxiety or depression scores in either treated group except BDI depression score which showed a positive correlation with a percentage decrease of MCP-1 and TNF-α ($p < 0.01$). LD-1227 and Omega-3 comparably reduced the level of IL-6 whereas the omega-3 treatment did not affect the level of TNF-α. Moreover, unlike omega-3, LD-1227 significantly reduced the other inflammatory cytokines ($p<0.05$) and was the only one normalizing IL-13 ($p<0.05$).

Table 2. Cytokines profile after 3 months of treatment with LD-1227 or Omega-3

Biomarker	LD-1227 (12 weeks)	Omega-3 (12 weeks)	Statistics LD-1227 vs omega 3
IL-6 (pg/ml)			
At entry	2.2±0.2*	2.1±0.4*	ns
End of study	1.5±0.4§	1.7±0.4§	ns
Control 1.5±0.3			
TNF-α (pg/ml)			
At entry	23.3±6.1*	22.1±4.1*	ns
End of study	16.5±4.4**	20.7±3.6	< 0.01
Control 14.4±5.3			
IL-1β (pg/ml)			
At entry	4.6±0.3*	4.9±0.2*	ns
End of study	2.5±0.2**	4.5±0.6	< 0.01
Control 2.2±0.3			
IFNγ (pg/ml)			
At entry	2.4±0.3	2.6±0.4	ns
End of study	2,2±0.2	2,5±0.3	ns
Control 3.1±0.4			
MCP-1 (pg/ml)			
At entry	202.1±23.1*	196.6±21.1*	ns
End of study	172.7±8.1§	179.8±7.8§	ns
Control 162.2±8.3			
IL-8 (pg/ml)			
At entry	188.1±85.1*	181.2±65.2	ns
End of study	74.1±34.1	174.8±31.6	< 0.01
Control 72.2±24.3			

Biomarker	LD-1227 (12 weeks)	Omega-3 (12 weeks)	Statistics LD-1227 vs omega 3
IL-1α (pg/ml)			
At entry	1.4±0.1	1.3±0.2	ns
End of study	1.3±0.6	1.3±0.2	ns
Control 1.2±0.2			
IL-18 (pg/ml)			
At entry	264.8±65.7	266.9±61.9	ns
End of study	252.9±44.4	259.5±43.4	ns
Control 246.5±26.7			
hs-CRP (mg/L)			
At entry	0.9±0.2*	0.8±0.2*	ns
End of study	0.5±0.3**	0.7±0.3	< 0.05
Control 0.4±0.1			
GM-CSF(pg/ml)			
At entry	3.5±0.2	3.4±0.4	ns
End of study	3.4±0.4	3.3±0.4	ns
Control 3.3±0.3			
IL-13 (pg/ml)			
At entry	1.27±0.2*	1.31±0.4*	ns
End of study	1.51±0.1**	1.36±0.3	< 0.01
Control 1.60±0.4			

Cytokines profile at entry and 3 months afterward, following either LD-1227 or omega-3 treatments. Healthy subjects served as healthy control. *p<0.05 vs healthy control; **p<0.05 vs entry level.

Fig. 1

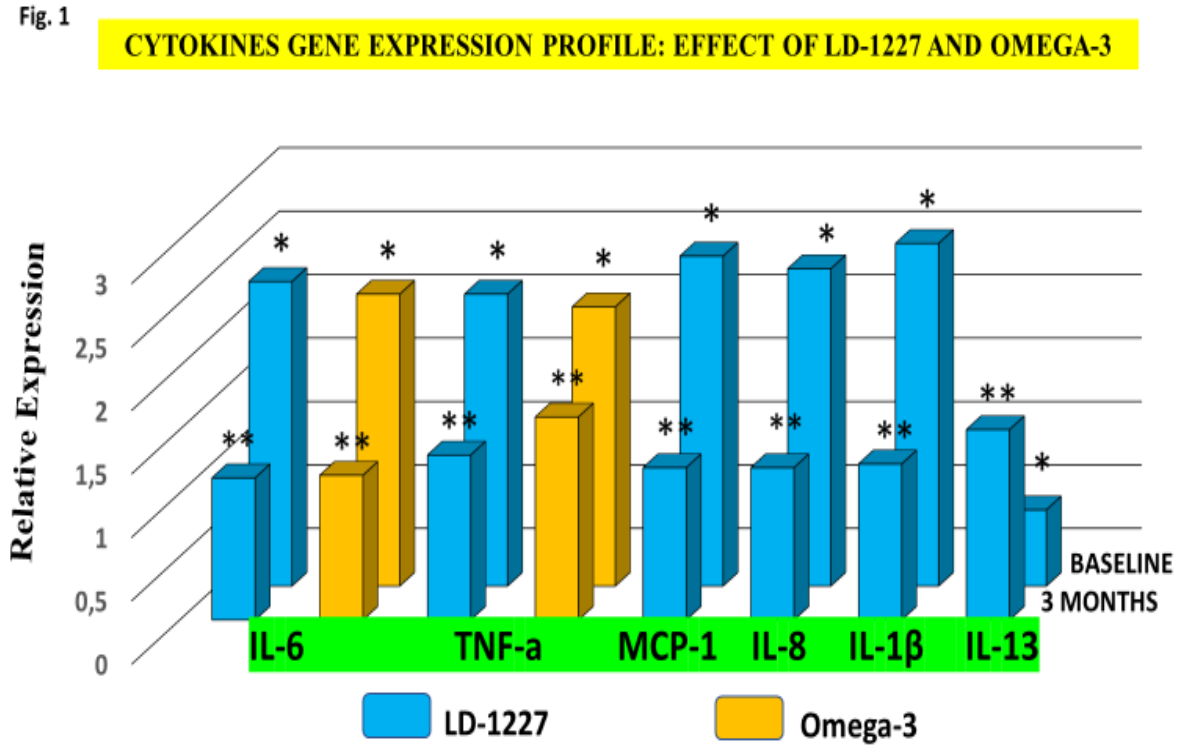


Figure.1. Gene expression of cytokines which showed altered level in the serum. *p<0.01 vs reference gene arbitrarily set as 1 in x axis; **p<0.05 vs entry level. LD-1227 and omega-3 comparably downregulated IL-6 and TNF-alpha. Only LD-1227 significantly modified the other genes (see the text).

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