
T helper 1 response is correlated with widespread pain, fatigue, sleeping disorders and the quality of life in patients with fibromyalgia and is modulated by hyperbaric oxygen therapy

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ABSTRACT

Objective. Hyperbaric oxygen therapy (HBOT) has been used as treatment for different clinical conditions, including fibromyalgia (FM). HBOT modulates brain activity, ameliorates chronic pain and modifies the ratio of immune cells. Clinical studies have provided evidence that FM is associated with immune system dysregulation. In the present study we aimed to evaluate the effect of HBOT on immune system and on the quality of life-style of FM patients.

Methods. Patients with primary FM and controls were treated with HBOT. Physical, emotional and social assessment, quality of sleep, tender points, intensity score, WPI and symptom severity were evaluated before and after HBOT. Furthermore, a characterisation of CD4 T lymphocytes and their cytokine production was performed by flow cytometry. The expression of TNF- α , IFN- γ , IL-17, IL-9 and IL-22 was also assessed by RT-PCR. Finally, the serum levels of serotonin were evaluated by ELISA.

Results. Our results confirm the participation of immune system in the pathogenesis of FM and highlight the impact of HBOT treatment, with particular regard to the changes on proinflammatory cytokines production by CD4 T cells subsets.

Conclusion. FM patients show a Th1 signature and the activation of this subset is modulated by HBOT.

Introduction

Fibromyalgia syndrome (FM) is a very common disorder, often associated with other rheumatic diseases (*i.e.* spondyloarthritis, connective tissue disease, etc.), with a prevalence of 2 to 4% (1, 2). It is a chronic and diffuse

musculoskeletal disease affecting prevalently women (9:1 ratio woman/man), without any evidence of gender-related mechanism (3).

FM is characterised by a number of common but non-specific symptoms: chronic widespread pain with concomitant fatigue, sleep disturbance, irritable bowel syndrome, headache and mood disorders; in addition, cognitive impairment as alteration of short-term memory consolidation, speed of information processing, attention span and multitasking activities (4) are considered the hallmarks of this clinical condition (5). The daily activities appear difficult for about 50% of FM patients and 30-40% of them experience failure to do their job (6), and require significant and permanent lifestyle changes.

The aetiology of FM is still not clear: there is not a single trigger for FM and a lot of physical and/or emotional conditions can trigger or worsen symptoms (7, 8). Despite the well-known connection between the nervous system and its mediators (such as serotonin) and immune cells, information on distribution of lymphocyte subsets under stress and pain conditions is limited (9). Almost all immune cells express at least one serotonin receptor and several immunoregulatory functions (*i.e.* modulation of cytokine secretion, T-cell activation, etc.) have been ascribed to serotonin in recent years (10).

Pro-inflammatory cytokines (*i.e.* IL-1RA, IL-6, and IL-8 but not IL-5, IL-4, IL-13) have been already described as involved in the pathogenesis of FM but so far only few data are present in literature regarding their role (11).

Currently FM treatment is focused on combination of non-pharmacological and pharmacological interventions (6)

directed to reduce pain and improve the quality of life. Recently, hyperbaric oxygen therapy (HBOT) has been used as treatment for different clinical conditions according to Hyperbaric Medicine Society's indications (12, 13). There are evidences of the analgesic effects of HBOT in nociceptive, inflammatory and neuropathic pain models in mice (14, 15) and humans (16). Several studies have demonstrated that HBOT modulates brain activity, ameliorates chronic pain and modifies the ratio of immune cells (12, 17). An earlier study demonstrated a significant reduction in tender points and VAS pain and a significant increase in pain threshold after HBOT in FM patients (18).

In the present study we have characterised immune cells in the peripheral blood of FM patients and evaluated the effect of HBOT on immune system and on health-related quality of life, with the aim of better understanding the pathogenesis of the disease.

Material and methods

Patients

Thirty-six patients (34 females and 2 males, mean age 38±12 years, mean disease duration 34±12 months) with primary FM, who fulfilled the 1990 American College of Rheumatology classification criteria (19) and the 2010 American College of Rheumatology preliminary diagnostic criteria (20) and 10 age- and sex-matched healthy controls (Controls) were recruited at Policlinico Paolo Giaccone University Hospital, Palermo, Italy and enrolled in this study. Ten patients without systemic inflammatory disorders (decompression sickness, sudden and painless vision loss) (CTR HBOT) that underwent HBOT treatment were also enrolled as unrelated disease controls. Patients with other autoimmune disorders and secondary FM, acute infectious diseases in the previous 3 weeks were excluded from the study. Fourteen patients were not suitable for HBOT and were considered as disease (FM) control group, twenty-two were found eligible for HBOT and were treated accordingly with the following HBOT protocol: 40 daily sessions, 5 days/week, 90 minutes each, 100% oxygen with air breaks at 2.0AT.

Table I. Demographic characteristics of patients with fibromyalgia syndrome (FM) and controls.

	FM HBOT T0 n=22	FM untreated group n=14	Healthy controls (controls) n=10	Unrelated disease control group (CTR HBOT T0) n=10
Sex (F:M)	20:2	14:0	9:1	8:2
Age (years, mean ± SD)	36 ± 8	38 ± 10	37 ± 7	39 ± 4
Disease duration (years, mean ± SD)	38 ± 12	38 ± 6	-	-
BMI (mean ± SD)	25.4 ± 5.7	24.8 ± 6.5	25.7 ± 4.3	24.3 ± 7.1
Smoking	18%	14%	10%	20%
Depression	45%	42%	10%	10%
Educational level:				
primary school	42%	44%	46%	40%
secondary school	37%	38%	32%	36%
high school / university	21%	18%	22%	24%
Number of comorbidities				
0	36%	32%	39%	30%
1	41%	50%	51%	59%
≥2	33%	18%	10%	11%

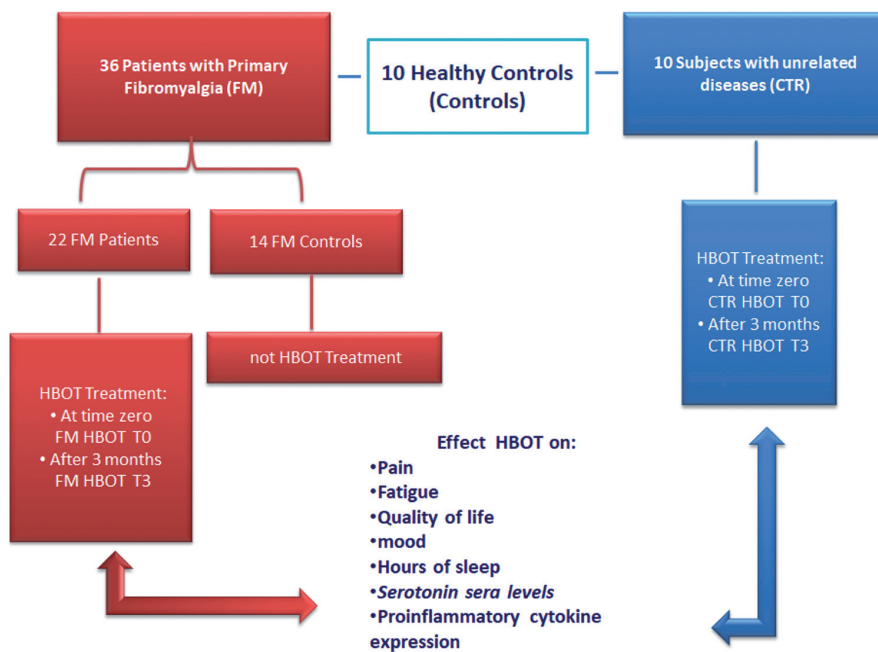


Fig. 1. Flow chart of the study.

Exclusions criteria for HBOT were chest and heart pathology, inner ear disease, claustrophobia and smoking. Patients and controls baseline characteristics, collected at the first interview, are shown in Table I. Study design is represented in Figure 1. Informed consent was obtained from all participants. The study was approved by the local University Hospital ethics committee.

Physical, emotional and social assessment

Widespread pain (WP), fatigue, mood and sleeping disorders and the quality of

life were assessed at time 0 (T0) and after 3 months (T3) of HBOT, with tender points count, visual analogue scale (VAS) pain, intensity pain score, visual analogue scale (VAS) fatigue, Widespread Pain Index (WPI), symptom severity (SS), Mood score, Health Assessment Questionnaire (HAQ) score, Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT fatigue) scale and the hours of sleep per day, respectively.

Tender points count and intensity pain score

Manual Tender Points Examination was assessed for number and severity as

described in the 1990-ACR guidelines (19). Digital pressure of approximately 4kg was applied at each of the 18 predefined TP sites, and the patient's pain response at each site was scored between 0 = no pain to 10 = severe pain.

Visual Analogue Scale (VAS) pain,

Visual Analogue Scale (VAS) fatigue

The VAS is a straight, 100-mm line (10 cm) that represents continuous pain or fatigue intensity, where the left end of the line indicates "no pain," and/or "no fatigue" while the other end denotes "pain or fatigue as bad as it could possibly be." A patient indicated their level of pain (in mm), by marking a single point on a line (21).

Widespread Pain Index (WPI)

It indicates the number areas (accordingly to the new preliminary diagnostic ACR criteria of FM) in which the patient has had pain over the last week. The score will be between 0 and 19 (20). The WPI was assessed by physician

Symptom Severity Scale (SS)

This scale is based on the patient's experienced severity of fatigue, waking unrefreshed and cognitive symptoms. The patient indicated whether, during the past week, they have experienced these symptoms and the score 0= no problem, 1= slight or mild problem, 2= Moderate problem, 3= severe problem. (20).

Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT fatigue)

The FACIT Fatigue Scale is a short questionnaire, 13 items, that measures an individual's level of fatigue during their usual daily activities over the past week. The level of fatigue is measured on a four point Likert scale (4 = not at all fatigued to 0 = very much fatigued) (22).

Health Assessment Questionnaire (HAQ) score

It includes 8 sections: dressing, arising, eating, walking, hygiene, reach, grip, and activities. There are 2 or 3 questions for each section. Scoring within each section is from 0 (without any difficulty) to 3 (unable to do).

The 8 scores of the 8 sections are summed and divided by 8 (23).

Pittsburgh sleep quality index - PSQI

This index is used to investigate the sleep quality level of the patient and to demonstrate any sleep disturbance during the previous month. It consists of 19 items and seven subscales called "sleep time", "sleep disturbances", "sleep latency", "daytime functionality loss", "habitual sleep efficiency", "subjective sleep quality" and "use of sleeping medication". Each item is weighted on a 0-3 interval scale. The global PSQI score is then calculated by totaling the seven component scores, providing an overall score ranging from 0 to 21, where lower scores denote a healthier sleep quality (24).

Isolation of peripheral blood mononuclear cells and flow cytometry

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) from 22 FM patients, 10 healthy controls and 10 patients with non-related autoimmune disorders that underwent HBOT treatment. Cell viability (trypan blue dye exclusion) was always >95%. Cells cultured in RPMI 1640 (Euroclone, MI, Italy) supplemented with 10% FCS and antibiotics (Euroclone, MI, Italy) were activated with anti-CD3/CD28 beads (ratio 1:2) and/or ionomycin (Sigma, St. Louis, MO, US 1 µg/mL final concentration) and phorbolmyristate acetate (PMA, Sigma, 150ng/mL final concentration) for 6h in the presence of 10 mg/ml of monensin (Sigma, St. Louis, MO) for blocking protein secretion, and then stained with appropriate mAbs. Flow cytometry analysis was performed using a FACSCanto (BD Biosciences). At least 100,000 cells (events), gated on lymphocytes region, were acquired for each sample. Data were analysed with FlowJo software programs. The antibodies used were the following: PE anti-human TNF-α (clone MAb11, Biologend, San Diego, CA, USA), APC anti-human IFN-γ (clone 4S.B3, Biologend), APC anti-human IL-22 (clone 2G12A41, Biologend), PE anti-human IL-17 (clone eBio 64CAP17, eBioscience Affimetrix Inc., San Diego, CA, USA), Alexa Fluor 647 anti-human

IL-9 (clone MH9A4, Biologend), PE anti-human PU-1 (R&D Systems, Minneapolis, MN, USA), PerCP/Cy 5.5 anti-human CD3 (clone UTCHT1, Biologend), Pe-Cyanine 7 anti-human CD8a (clone SK1, eBioscience Affimetrix Inc), PerCp anti-human CD4 (MEM-241, Abcam, Cambridge UK).

RT-PCR

Total RNA was isolated from PBMC of FM patients and controls at baseline and after HBOT using the commercially available RNeasy RNeasy Spin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For quantitative TaqMan RT-PCR, master mix and TaqMan gene expression assays for GAPDH (Hs99999905_m1) housekeeping, for TNF-α (Hs00174128_m1), IFN-γ (Hs00989291_m1), IL-17 (Hs00174383_m1), IL-22 (Hs01574154_m1) were obtained from Applied Biosystems. Samples were run in triplicate using the Step-One RT-PCR System (Applied Biosystems). Relative changes in gene expression between patients and controls at T0 and T3 were determined using the $\Delta\Delta C_t$ method. Final values were expressed as fold induction.

Enzyme-linked immunosorbent assay (ELISA) for TNF-α and IFN-γ
Cytokine production from sera of patients and controls was assayed by commercial ELISA (Abcam) according to manufacturer instructions.

Enzyme-linked immunosorbent assay (ELISA) for serotonin
Serotonin production from sera of patients and controls was assayed by commercial ELISA (Abcam) according to manufacturer instructions.

Statistical analysis

Presentation of sample size is based on achieving an overall 80% power to demonstrate that improvement rate in tender sites is at least 0.50 (these improvements were based on the improvements

reported by Efrati *et al.* 2015). Assuming a true success rate of 87% a sample of n=31 will provide 80% power to show that HBOT treatment induces at least 87% improvement on tender sites. This is based on a power analysis using the normal approximation for the binomial, with one-sided alpha=0.05. Student's *t*-test, ANOVA and a multivariate linear regression were used, when appropriated, to calculate the statistical significance between groups. Adjustment of *p*-value due to multiple outcomes comparison has been performed by using Sedak test and more conservative Bonferroni test adjustment. *p*-values less than 0.05 were considered significant.

Results

Effect of HBOT on pain, fatigue,

quality of life, mood and hours of sleep

The effect of the HBOT on patients' pain, was assessed by the evaluation of changes in the number of tender points, in the value of intensity score pain, of WPI and of symptom severity. As shown in Table II both the two groups (FM HBOT T0 and FM untreated) had very close mean scores at baseline for all the measures. HBOT treatment of FM patients led to statistically significant improvements in the mean scores of pain perception. Particularly, as shown in Table III a significant reduction in the number of tender points, in the value of intensity score pain, of WPI and of symptom severity was found in FM HBOT-treated patients (FM T0 vs. FM T3).

We also evaluated HBOT effects on the physical functions, the psychological distress and the quality of life among FM HBOT-treated patients (T3). As shown in Table II and Table III, a statistically significant improvement was found in the FACIT questionnaires. HAQ questionnaires was found significant only at 10%. On the contrary HBOT did not improve the total hours of sleep, but as referred by patients the quality of sleep ameliorates in FM patients after the treatment (Table III).

Serotonin sera levels are reduced in

FM and correlate with disease severity

Serotonin levels in the sera of FM patients at T0 (minimum 20 ng/ml,

Table II. Mean scores at baseline (T0) and after HBOT (T3).

	FM HBOT T0 n=22	FM HBOT T3 n=22	FM untreated n=14
Widespread Pain Index (mean ± SD)	14.8 ± 0.7	10.9 ± 0.9	15 ± 1.2
Symptom Severity (mean ± SD)	9.19 ± 0.5	5.5 ± 0.5	9.0 ± 0.2
Tender points (mean ± SD)	16.41 ± 0.6125	13.5 ± 0.6	15.3 ± 0.9
VAS pain (mean ± SD)	7.7 ± 0.3	6.2 ± 0.4	8 ± 0.2
VAS fatigue (mean ± SD)	8.2 ± 0.3	6.4 ± 0.4	8 ± 0.4
Mood score (mean ± SD)	2.3 ± 0.3	1.7 ± 0.2	2.2 ± 0.2
HAQ (mean ± SD)	17.9 ± 2.5	13.7 ± 2.1	17.3 ± 3.1
FACIT (mean ± SD)	15.8 ± 1.6	28.3 ± 2.1	16 ± 1.1
Hours of sleep (mean ± SD)	4.7 ± 0.2	5.4 ± 0.3	5.1 ± 0.4
PSQI (mean ± SD)	18.7 ± 2.7	8.8 ± 1.0	3 ± 1.8

Table III. HBOT effects on the physical functions, the psychological distress and the quality of life.

FM T0 vs. FM T3	Mean diff	SE	Sidak's test Adjusted <i>p</i> -value	Bonferroni's test Adjusted <i>p</i> -value
FACIT	-12.43	1.916	0.0000179	1.79E-05
Symptoms severity	3.619	0.603	0.0000583	6.56E-05
PSQI	9.636	1.223	0.0000942	0.000121
Tender points	2.863	0.574	0.0003745	0.000562
WPI	3.909	0.884	0.0011974	0.002156
Intensity score	2.111	0.580	0.0075033	0.01693
HAQ	4.181	1.954	0.084092	0.398804
Hours of sleep	-0.681	0.304	0.104092	0.323785
Mood scale	0.545	0.299	0.104092	0.746078

maximum 87 ng/ml) were significantly lower compared to the control group (healthy controls) (*p*=0.001; minimum 111 ng/ml, maximum 302 ng/ml). Interestingly, the level of serotonin in FM patients increased after HBOT treatment (Fig. 2 A) but they did not correlate either with an improvement in the mood score (data not shown) or with the FACIT score (Fig. 2B).

mRNA expression proinflammatory cytokines

In order to investigate cytokine production and their potential association with specific pattern of inflammation, we first performed a RT-PCR analysis for proinflammatory cytokines among PBMCs from FM patients and healthy controls (Controls). As shown in Table IV, strong basal expression of TNF-α and IFN-γ mRNAs -, but not IL-17A and IL-22 mRNAs, was observed in FM patients respect to healthy controls, suggesting a proinflammatory status in FM characterised by the activation of genes related to Th1-type responses. Furthermore, HBOT treatment caused a profound and significant reduction of

all cytokines mRNAs in all the tested patients (Table IV). The mRNA expression level of TNF-α was directly correlated with the number of tender points (*r*2 = 0.54, *p*<0.0025) and the HAQ (*r*2 = 0.53, *p*<0.001) (data not shown).

TNF-α and IFN-γ serum levels are increased in FM patients and correlate with disease severity

Serum levels of TNF-α and IFN-γ were also measured and were found significantly higher in FM compared to healthy controls (Controls) (*p*<0.001 for both) at baseline (Table V). Of note, TNF-α and IFN-γ serum levels correlated significantly with symptom severity score (*r*2=0.51 for TNF-α *p*< 0.0001 and *r*2=0.54 for IFN-γ, *p*<0.0025) (Fig. 2C-D) The levels of these cytokines dramatically dropped out after HBOT treatment (Table V).

Percentage and cytokine production by T lymphocytes at baseline and after HBOT

Since HBOT shows immunomodulatory capability, we evaluated a possible effect on the immune cell response. We

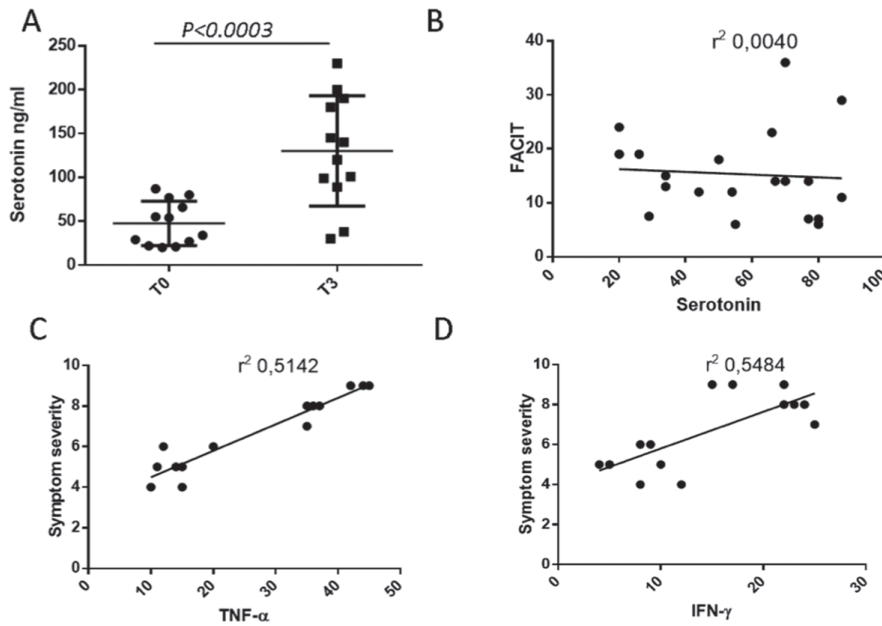


Fig. 2. HBOT effects on the physical functions, the psychological distress and the quality of life. **A.** Mean relative changes in serum serotonin level at T0 and T3. **B.** Correlation between FACIT and serotonin sera levels. $P<0.05$ were considered significant. **C-D.** Correlation between symptom severity and TNF- α (C), IFN- γ (D) sera levels respectively.

Table IV. Differences of mRNA mean expression.

FM vs. Controls	Mean diff	SE	Sidak's test Adjusted p -value	Bonferroni's test Adjusted p -value
IFN- γ	0.92333	0.01453	0.00099	0.00099
TNF- α	0.93333	0.01666	0.00099	0.00127
IL-17	0.86211	0.07023	0.01316	0.02641
IL-22	0.85012	0.07637	0.01316	0.03190
FM T0 vs. FM T3				
IFN- γ	0.28333	0.070317	0.03951	0.040109
TNF- α	4.29166	1.121786	0.03951	0.049204
IL-17	-0.12167	0.26806	0.66893	2.675751
IL-22	-0.44167	0.156214	0.07222	0.147162

used flow cytometry to analyse circulating T cell subsets in patients (FM and patients with unrelated diseases control group: CTR HBOT) before and after HBOT treatment (T0, T3).

As shown in Fig. 3A-B, no differences were found in the mean percentage of total CD4 and CD8 lymphocytes at baseline and after HBOT both in patients (T0: CD4 50%, CD8 23%, T3: CD4 48%, CD8 35%) and CTR HBOT (T0: CD4 42%, CD8 23%, T3: CD4 37%, CD8 22%). Additionally, no significant differences were found between CTR HBOT T0 and healthy controls (Controls) (CD4 40%, CD8 21%, T3: CD4 45%, CD8 25%) (data not shown). We then analysed cytokine production among T cell population. As T cells

immediately secrete cytokines *in vivo*, intracellular detection of cytokines in these cells *ex vivo* normally requires the incubation with brefeldin A or monensin, which block protein secretion. Thus, PBMCs were shortly incubated in the presence of monensin. In single-colour analyses among T cells, the percentages of total lymphoid cells expressing IL-17A, IL-22, IL-9, TNF- α and IFN- γ was compared in FM patients (T0) and CTR HBOT (T0). As shown in Figure 3C-D, no significant differences were found among FM patients and CTR HBOT. (IL-17 FM vs controls (CTR HBOT) 0.04 ± 0.06 vs. $0.01\pm 0.009\%$, $p>0.05$; IL-22 0.34 ± 0.39 vs. $0.06\pm 0.02\%$, $p>0.05$; IL-9 0.27 ± 0.40 vs. $0.5\pm 0.09\%$, $p>0.05$ and

IFN- γ 0.7 ± 0.62 vs. 0.20 ± 0.08 $p>0.05$, respectively).

In contrast, the analysis of CD4⁺ T cell subsets (able to produce several cytokines) showed different expression of IL-17A, IL-22, IL-9, TNF- α and IFN- γ in FM patients and CTR HBOT at T0. Particularly, a Th1-type signature (TNF- α and IFN- γ) was found in FM patients respect to CTR HBOT before treatment (Th17 FM vs. CTR HBOT 0.07 ± 0.09 vs. $0.07\pm 0.08\%$, $p>0.05$; Th22 0.30 ± 0.34 vs. $0.07\pm 0.5\%$, $p>0.05$; Th-9 0.14 ± 0.11 vs. $0.36\pm 0.08\%$, $p>0.05$ and Th1 IFN- γ 0.99 ± 0.62 vs. 0.11 ± 0.13 , $p<0.05$, Th1 TNF- α 0.40 ± 0.34 vs. $0.5\pm 0.33\%$, $p<0.05$, respectively) (Fig. 4A-B). Additionally, in the presence of Ionomycin and PMA, there was a significant increase of TNF- α and IFN- γ expression in CD4⁺ T cells of FM patients respect to CTR HBOT, Th1 IFN- γ 1.64 ± 1 vs. $0.97\pm 0.3\%$, $p<0.05$; Th1 TNF- α 15.8 ± 1.6 vs. 12 ± 8 , $p<0.05$) (Fig. 4C-D).

CD4 T cells were then analysed for simultaneous expression of cytokines. Circulating IL-17A⁺IL-22⁺ double positive cells were not expanded in FM patients, when compared with CTR HBOT (FM vs. CTR HBOT IL17+/IL22+ 0.016 ± 0.03 vs. $0.009\pm 0.013\%$, $p>0.05$) (data not shown), conversely, TNF- α ⁺IFN- γ ⁺ double positive cells were increased in FM after Ionomycin PMA stimulation when compared to CTR HBOT (5.23 ± 1.2 vs. 0.53 ± 0.08 , $p>0.05$) (Fig. 4C-D), indicating expansion of Th1 but not Th17/Th22 cells. Interestingly, IL-17A and IL-22 were expressed by different cells, suggesting a Th22 polarisation. Circulating CD4⁺ cells negative for all these cytokines were significantly lower in FM patients than in controls at baseline (41% vs. 52%), indicating a general trend toward higher levels of inflammatory cytokine-producing CD4⁺ cells in subjects with FM compared with those without disease (Supplementary Fig. 1A).

Finally, we evaluated if the distribution of lymphocyte subsets was affected by HBOT. Twenty-two patients and 10 CTR under HBOT (CTR HBOT) were evaluated after three months (T3) of treatment. HBOT over time led to

Table V. Multivariate linear regression model of TNF- α and IFN- γ .

VARIABLES	FM and controls				FM T0 and FM T3			
	TNF- α coef	se	IFN- γ coef	se	TNF- α coef	se	IFN- γ coef	se
FM vs. Controls	24.9235***	2.12	10.9708***	1.70				
FM T0 vs. FM T3					-0.0368***	0.00	0.4569***	0.09
Depression	1.1190	2.11	-1.6456	1.70	0.0855	0.17	-6.4847	4.74
BMI	0.0660	0.22	0.2166	0.18	-0.0073	0.01	0.5244	0.36
Smoking	-1.2969	2.28	0.8488	1.83	-0.1338	0.16	2.8997	4.53
Constant	13.0651**	5.07	5.2401	4.07	1.6419***	0.30	-8.4011	8.49
Observations	32		32		44		44	
R-squared	0.807		0.769		0.831		0.737	

*** $p < 0.01$.

a massive and significant reduction of all tested (17A, IL-22, IL-9, TNF- α and IFN- γ) and particularly of the Th1-related cytokines TNF- α and IFN- γ . The decrease of this Th1 cytokine response was correlated with a global improvement in disease scores in all patients. No significant modifications were found for the other T cells subsets at T3 (Supplementary Fig. 1B).

Discussion

The analgesic effects of HBOT have been studied in nociceptive, inflammatory and neuropathic pain models, and it was demonstrated to be useful for the treatment of various chronic pain syndromes including FM (25). Herein we have evaluated the clinical response to HBOT treatment in our cohort of FM patients in modulating pain thresholds,

fatigue, cognitive field, mood disturbance and the quality of sleeping. Our results confirm the participation of immune system in the pathogenesis of FM and highlight the impact of HBOT treatment, with particular regard to the changes on proinflammatory cytokines production by CD4 T cells subsets. FM is one of the most common causes of chronic widespread pain among

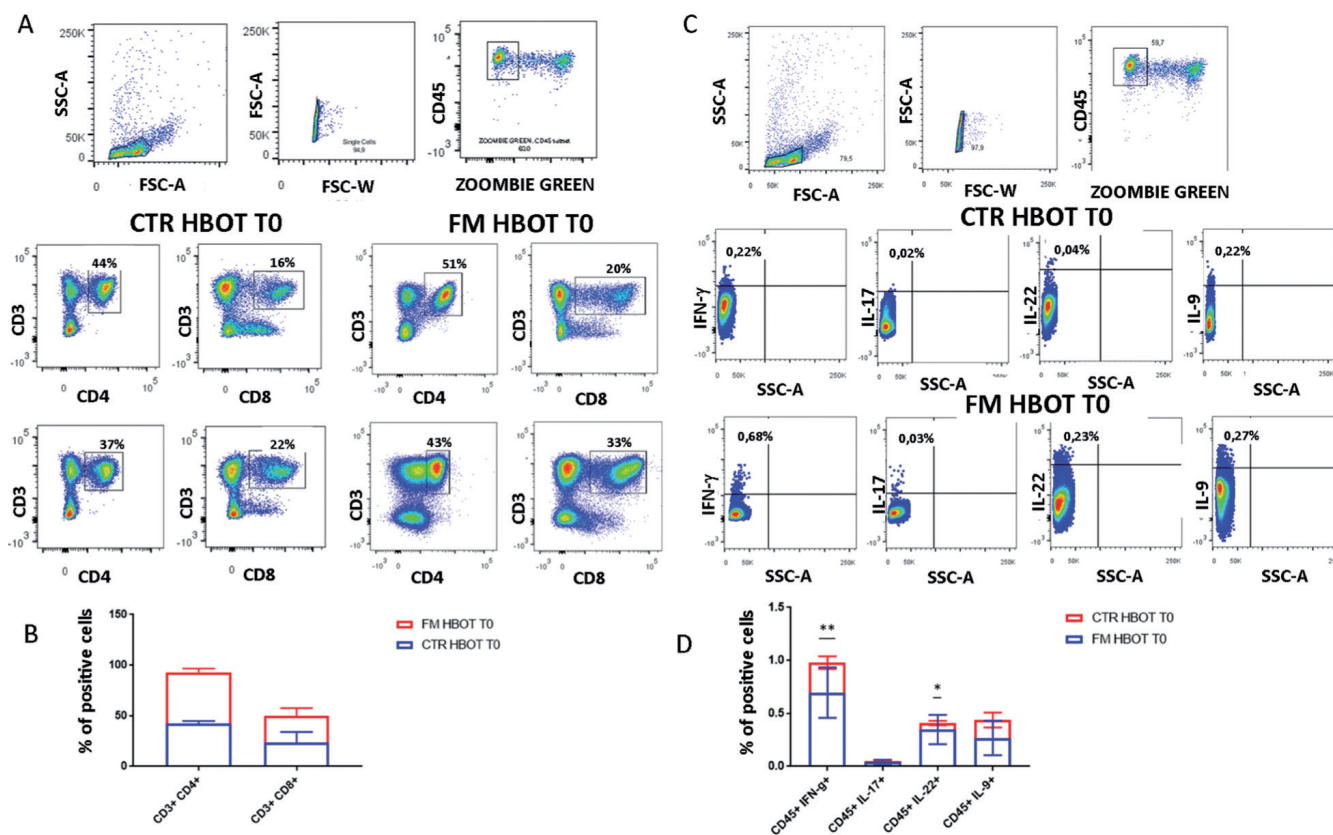


Fig. 3. A. Representative dot plot analysis of CD4 and CD8 lymphocytes from one FM patient and one CTR HBOT at T0. Mean percentage of CD4 and CD8 changes in FM and CTR HBOT at T0. C. Representative dot plot analysis of cytokines expression among CD45+ cells in one FM patient and one CTR HBOT at T0. D. Mean percentage of cytokines expressing CD45+ cells in FM and CTR at T0.

* $p < 0.05$; ** $p < 0.01$.

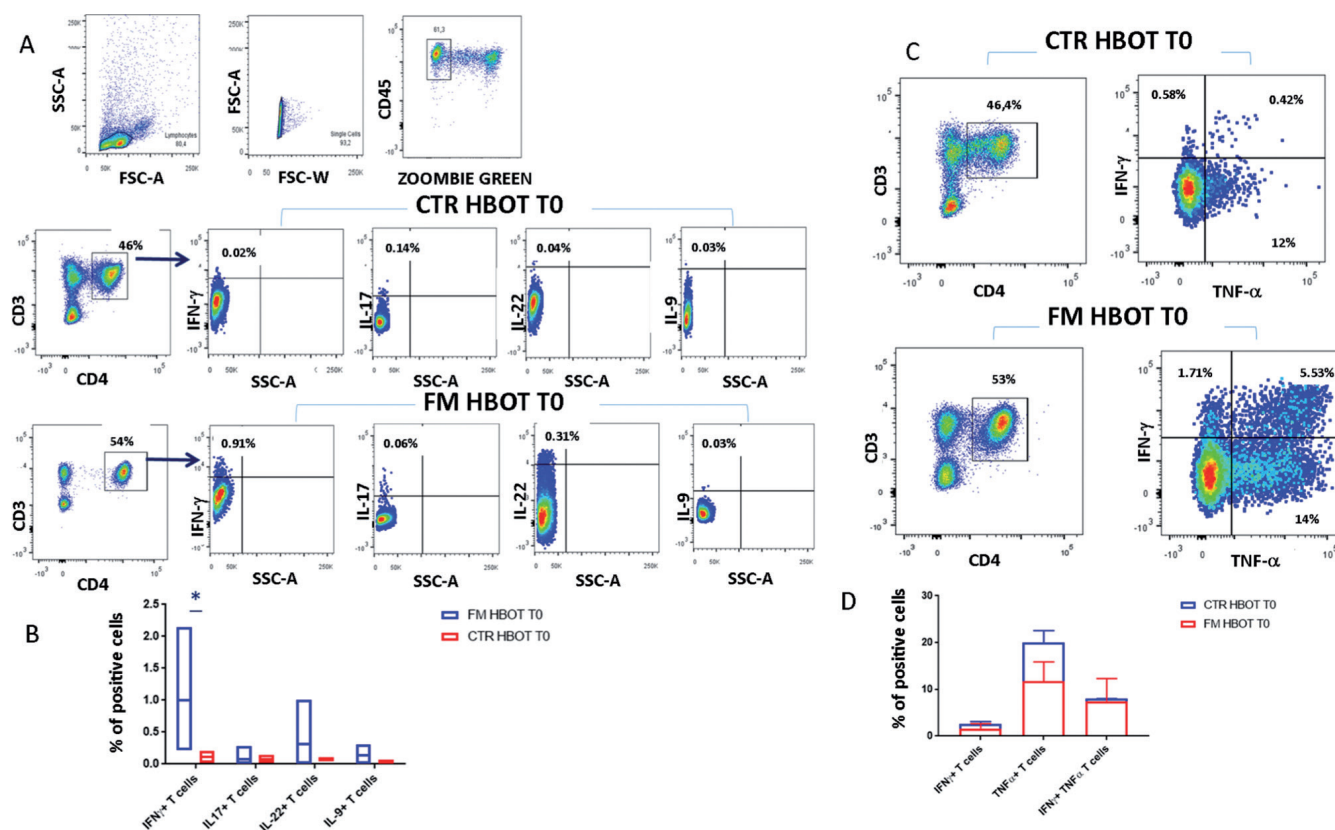


Fig. 4. **A.** Representative dot plot analysis of cytokines expression among CD3⁺ CD4⁺ cells in one FM patient and one CTR HBOT at T0. **B.** Mean percentage of cytokines expressing CD4⁺T cells in FM and CRT at T0. **C.** Representative dot plot analysis of cytokines expression among CD3⁺ CD4⁺ cells after IONO+PMA stimulation in one FM patient and one CTR HBOT at T0. **D.** Mean percentage of cytokines expressing CD4⁺T cells in FM and CTR HBOT at T0. **p*<0.05.

rheumatic diseases. It is characterised by reduced pain thresholds, fatigue, non-refreshed sleep, mood disturbance and cognitive impairment (5, 26). The management of chronic pain is sometimes challenging and requires a multidisciplinary approach. Most pharmacological and physical therapies only slightly or temporarily ameliorate pain symptoms (27).

According to data present in the literature, our FM patients showed a very high pain score and fatigue that affect the carrying out of daily activities (6). The restful sleep associated with awakening headache greatly contribute to aggravating the clinical picture of FM patients (5). In view of the enormous set of symptoms and the constant chronic pain poorly responsive to traditional therapies, a new therapeutic approach has already been considered in patients with chronic pain and more recently with FM. In fact, several studies have already demonstrated that HBOT can modulate chronic pain changing

pain perceptions by the induction of the neural nitric oxide-dependent release of opioid peptides (28). This condition may explain the improvement of pain threshold, symptom severity, sleep and quality of life also in FM patients. Yildiz *et al.* have already found that HBOT significantly reduced the number and threshold of tender points (18), and an Israeli group evaluated its efficacy in improving the symptoms of fibromyalgia patients by rectifying their typically altered brain functions (25). Particularly, Serotonin [5-hydroxytryptamine (5-HT)] a neurotransmitter regulating sleep, appetite, mood and other important brain functions was demonstrated to be able to regulate many other organ systems acting as a peripheral hormone. In fact, most of the peripheral body's serotonin is circulating in the bloodstream, transported by blood platelets (29). Platelets store serotonin at very high concentrations in their dense granules (at 65 mM) and secrete it upon activation (30). Rest-

ing plasma serotonin concentrations (around 10 nM) can rapidly increase to 10 μM or more when platelets become activated at the site of thrombus formation or inflammation (31, 32).

Thus, although the levels of serotonin in the sera of patients with fibromyalgia appear to exhibit a tendency to be lower than in patients with unrelated disease controls and healthy controls (33), at least when measured by ELISA, the variation of serotonin levels also in our data (within the disease groups) after HBOT is not correlated with an improving in the mood of FM patients. This suggests that peripheral serotonin can have a major role in FM through the modulation of the inflammatory process and reduced level of peripheral serotonin can favour a proinflammatory status. Alterations in 5-HT signalling have been described in inflammatory conditions of the gut, such as inflammatory bowel disease. The association between 5-HT and inflammation, however, is not limited to the gut, as changes

in 5-HT levels have also been reported in patients with allergic airway inflammation and rheumatoid arthritis (34). Despite the described role of 5-HT in the activation of macrophages and DC in terms of production of proinflammatory cytokines and phagocytic activity, the activation of 5-HTR2B and 5-HTR7 has also been shown to promote anti-inflammatory macrophage polarisation suggesting the important role of serotonin in the modulation of immune response (35).

We therefore conclude that measurement of the concentration of serotonin in serum is not a useful tool for the evaluation of mood/depression but could be useful to evaluate the global inflammatory status of FM patients and their response to HBOT treatment since increased level of sera serotonin directly correlate with a good quality of life of FM patients.

Given the immunomodulatory role of serotonin we have characterised cytokine production in the peripheral blood of FM patients and the subsets of CD4 T lymphocytes often involved in the pathogenesis of immune-mediated disorders.

Differently from Kaufmann *et al.* (9) in our cohort of FM patients the CD4/CD8 ratio was found unmodified when compared to the control group.

Interestingly, we found an expansion of Th1 lymphocytes, characterised by increased expression of the Th1-related cytokines TNF- α and IFN- γ , indicating a Th1 signature in FM patients. Moreover, this proinflammatory status was dramatically modified during HBOT which contributes to a global improvement of the quality of life, pain perception and cognitive capacities.

Clinical studies have provided evidence that FM is associated with immune dysregulation of plasma levels of pro-inflammatory cytokines. Increase of cytokine production may provoke symptoms such as fatigue, fever, sleep, pain and myalgia (9), all of which occur in FM patients (36, 37). Alterations in pro-inflammatory cytokine levels have been observed in the serum and biopsies of FM patients (38, 39). Moreover, a pilot study has already demonstrated a possible pathogenetic

role of cytokines (TNF- α , IFN- γ , IL-1 and IL-6) in FM (40). Despite this evidence, another study has shown that treatment with anti-TNF- α monoclonal antibodies in patients with axSpA and coexisting FM does not improve pain perception and additionally, FM seems to have a negative impact on TNF- α response and retention rate (41). If on the one hand TNF- α blockade alone does not improve pain perception, but may lead to inadequate subjective assessment of the response to ongoing treatment of other immunomediated pathologies such as spondylarthritis, on the other HBOT treatment greatly enhances pain perception and consequently reduces the upregulated pro-inflammatory cytokines in patients with FM suggesting a pathogenic role for cytokines in FM.

The remarkable plasticity of CD4⁺ T cells allows individuals to respond to environmental stimuli in a context-dependent manner. A balance of CD4⁺ T cell subsets is critical to mount responses against pathogen challenges to prevent inappropriate activation, to maintain tolerance, and to participate in immune responses. CD4⁺ T cell subsets have significant cross-talk, with the ability to “differentiate” given appropriate environmental signals, suggesting that environmental factors could participate to the activation of immune system (42). Particularly, FM patients show a Th1 signature and the activation of this subset is modulated by HBOT.

Further studies are necessary to better characterise immune response underlying FM, to understand the possible pathogenetic mechanisms and to develop novel therapeutic tools.

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