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Article

Effect of Melatonin plus Zinc Supplementation on Fatigue Perception in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Randomized, Double-Blind, Placebo-Controlled Trial

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Abstract: Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex, multisystem, and profoundly debilitating condition, probably of multifactorial etiology. No effective approved drugs are currently available for its treatment. Several studies have proposed symptomatic treatment with melatonin and zinc supplementation in chronic illnesses; however, little is known about the synergistic effect of this treatment on fatigue-related symptoms in ME/CFS. The primary endpoint of the study was to assess the effect of oral melatonin plus zinc supplementation on fatigue in ME/CFS. Secondary measures included participants' sleep disturbances, anxiety/depression and health-related quality of life. A proof-of-concept, 16-week, randomized, placebo-controlled, double-blind trial was conducted in 50 ME/CFS patients assigned to receive either oral melatonin (1 mg) plus zinc (10 mg) supplementation (n = 24) or matching placebo (n = 26) once daily. Endpoint outcomes were evaluated at baseline, and then reassessed at 8 and 16 weeks of treatment and 4 weeks after treatment cessation, using self-reported outcome measures. Treatment was safe and well tolerated. The most relevant results were the significant reduction in the perception of physical fatigue in the active group at the final follow-up versus placebo ($p < 0.05$), and the significant improvement in the physical component summary at all follow-up visits in the experimental group. Our findings suggest that oral melatonin plus zinc supplementation for 16 weeks is safe and potentially effective in reducing fatigue and improving the quality of life in ME/CFS. This clinical study was registered on ClinicalTrials.gov (NCT03000777).

Keywords: chronic fatigue syndrome; fatigue; myalgic encephalomyelitis; melatonin; quality of life; sleep quality; zinc

1. Introduction

Myalgic encephalomyelitis, also termed chronic fatigue syndrome (ME/CFS) is a complex debilitating condition with no known etiology. Its hallmark symptoms are severe and disabling physical and mental fatigue linked to post-exertional malaise, which do not improve with rest and seriously interfere with work activity and daily life tasks. To date,

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no diagnostic tests or biomarkers have been established for ME/CFS, or any universally effective treatment exists [1].

Recently our group demonstrated a significant association between sleep quality, assessed through the Pittsburgh Sleep Quality Index (PSQI) questionnaire, and other clinical symptoms (fatigue, pain and psychopathology) using self-reported measures in patients with ME/CFS [2]. Beyond the diagnosis of ME/CFS, it is important to assess fatigue perception through the Fatigue Impact Scale (FIS-40) [3], quality of life through a health-related quality of life questionnaire (SF-36) [4], and anxiety and depression symptoms using the Hospital Anxiety and Depression Scale (HADS) [5]. The non-pharmacological treatment approaches of ME/CFS, based on scientific evidence, includes cognitive behavioral therapy and graded exercise therapy [6], and pharmacological treatment is administered to address the core symptoms such as pain, memory/concentration problems, anxiety and depression, orthostatic intolerance and sleep disturbances.

Alterations in redox metabolism homeostasis and bioenergetic status, and mitochondrial, neuroimmune and neurovegetative dysfunctions have been implicated in the etiopathogenic hypotheses of ME/CFS [7]. A detailed review of the literature suggests several marginal nutritional deficiencies, which may be of etiological relevance. These include deficiencies of several B-complex vitamins (primarily riboflavin, thiamine, and pyridoxine), vitamin C, magnesium, sodium, melatonin, zinc, L-tryptophan, L-carnitine, Coenzyme Q10, and essential fatty acids (omega-3 PUFAs such as EPA and DHA) [8-10].

Melatonin is an endogenous hormone whose concentrations vary according to the day/night cycle. It is produced primarily in the pineal gland, and participates in a wide variety of cellular, neuroendocrine, and neurophysiological processes. In addition to its hypnoinductive action, melatonin is a powerful antioxidant and reactive oxygen and reactive nitrogen species scavenger, reducing oxidative stress and inhibiting the formation of free radicals. It also stimulates the immune system; it has receptors on helper T lymphocytes and produces IL-4 which, in turn, triggers IgA production in B cells. It stimulates cytotoxic phagocytes and T lymphocytes and stabilizes circadian rhythms [11]. In this regard, our work group has demonstrated severe alterations in the circadian rhythm and autonomic dysfunction from ME/CFS patients [12]. Therefore, there is growing evidence that supports the possible use of melatonin in ME/CFS.

Zinc is an essential trace element, which is present in more than 300 specific metalloenzymes, playing an essential role in their activation or structural stability, in addition to serving as a structural ion in more than 200 transcription factors. Zinc plays a critical role in synaptic neuroplasticity and learning [13]. It is needed to achieve a balanced immune function, reducing pro-inflammatory cytokines and decreasing oxidative stress [14] and its use has been also shown to be effective in various depressive processes [15].

To date, symptomatic treatment using oral melatonin plus zinc supplementation in individuals with ME/CFS has not been evaluated. Effective treatment alternatives for ME/CFS are lacking, and the treatments available at present are mostly limited to providing partial symptom relief [6].

Nutraceutical supplementation is receiving increasing attention as a potential therapeutic intervention for chronic diseases. It has shown a good safety profile and promising clinical effects, but to date it has not been extensively assessed in ME/CFS [16,17]. Here we describe a first pilot trial to evaluate the clinical response after oral melatonin plus zinc supplementation on perception of fatigue, sleep disturbances, anxiety and depression and health-related quality of life in individuals with ME/CFS.

2. Materials and Methods

2.1. Study participants

A 16-week randomized, double-blinded, placebo-controlled pilot study was conducted at a single outpatient tertiary referral center (ME/CFS Unit, Vall d'Hebron University Hospital, Barcelona, Spain) from May 2016 (first patient inclusion) to August 2017 (last clinical visit). A total of 79 Caucasian Spanish ME/CFS were consecutively recruited

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over the course of the study. **Figure 1** shows a flowchart of the participants prior to analysis. Patients were eligible for the study if they were female, aged between 18 and 65 years, had a confirmed diagnosis of ME/CFS according to the 1994 CDC/Fukuda definition [18] and provided signed informed consent. Exclusion criteria were: any active medical condition that explained chronic fatigue (untreated hypothyroidism, sleep apnea, narcolepsy, medication side-effects), previous diagnosis not unequivocally resolved (chronic hepatitis, malignancy), past or current psychiatric disorders (major depressive disorder with psychotic or melancholic features, bipolar disorder, schizophrenia, delusional disorder, dementias, anorexia nervosa, bulimia nervosa), participation in another clinical trial of the same or different nature in the 30 days prior to study inclusion; in the judgment of the investigator, inability to follow the instructions or to complete the treatment satisfactorily; failure to provide signed informed consent; current consumption of medications that may interfere with the results, and or whose withdrawal may be a relevant problem, anticoagulant treatment, pregnancy or breast-feeding, smoking, alcohol intake or substance abuse, severe obesity (BMI > 45 kg/m^2), and hypersensitivity to melatonin and/or zinc. Patients with missing data from the follow-up visits to baseline were defined as having dropped out.

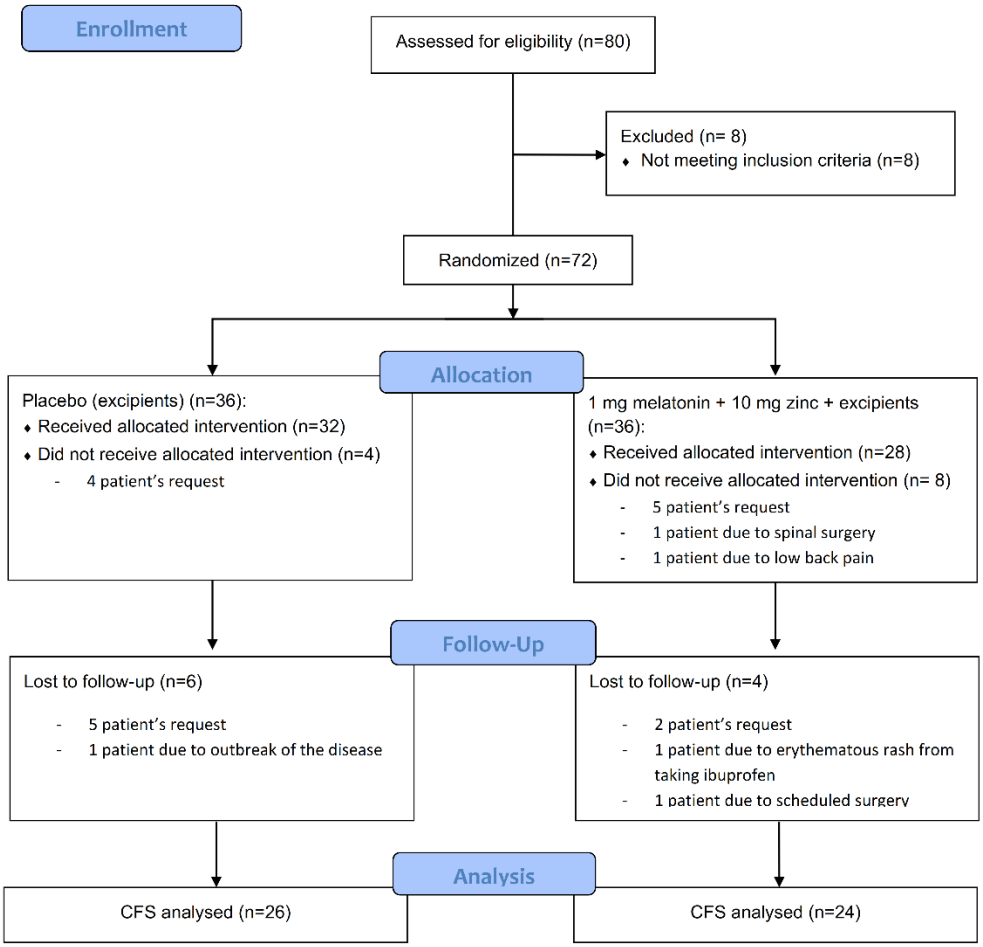

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flow diagram.

2.2. Intervention

Of the pre-selected patients, eight were excluded because they did not meet the inclusion criteria. Seventy-two participants were allocated to treatment by an independent investigator not otherwise involved in the intervention, using the table of random numbers in the Milton statistical guide [19]; they were assigned in a double-blind fashion in a 1:1 ratio either to melatonin plus zinc (n = 36) or matching placebo (n = 36) in capsules

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once daily at night (30 min before bedtime) for 16 weeks, in addition to the standard therapy. The study pharmacist recorded all treatments supplied on the medication-dispensing forms along with the original script.

2.3. Tested Product

The supplement tested contained a homogenized mixture of 1 mg melatonin plus 10 mg zinc including isomaltose, and magnesium stearate as excipient. The matching placebo capsules were made of the excipient only. The mixture was encapsulated in a purple gelatin capsule. Each bottle contained enough capsules for four months of consumption (120 capsules/bottle) at a rate of one capsule once a day. The preparations (active and placebo capsules) were identical in size, shape, color, opacity, and taste, and also in terms of presentation and packaging to avoid differentiation by the participants or the research staff. Both treatments were provided by Laboratorios Viñas, S.A. (Barcelona, Spain).

2.4. Study design

This 16-week randomized, double-blinded, placebo-controlled pilot trial was performed at a single outpatient tertiary referral center (ME/CFS Unit, Vall d'Hebron University Hospital, Barcelona, Spain) from May 2016 through August 2017. All participants were Caucasians, and had a sedentary lifestyle and from the same geographical area at the time of study. Clinical visits throughout the study are detailed in **Figure 2,** which also describes the trial design in both groups. After a verbal description of the study, all participants gave written consent prior to its commencement. Patients were evaluated at baseline, and each follow-up visit (after eight and 16 weeks of treatment and four weeks' post-treatment). Changes in symptoms were assessed through self-report questionnaires completed by participants under the supervision of two trained investigators (JC-M and JA) who oversaw compliance. The clinical study was registered at https://clinicaltrials.gov/ (reference number: NCT03000777).

Figure 2. Summarized study schedule at each visit throughout the clinical trial.

2.5. Primary outcome measure

The primary outcome measure was the evaluation of the relief of self-reported fatigue using the 40-item Fatigue Impact Scale (FIS-40) questionnaire after oral melatonin plus zinc supplementation in individuals with ME/CFS. The FIS-40 includes three subscales of the perceived impact of fatigue: cognitive (ten items), physical (ten items) and psychosocial functions (20 items), each item being scored from 0 (no fatigue) to 4 (severe fatigue). The total score is calculated by adding together responses from the 40 questions (range 0–160). Higher scores indicate more functional limitations due to fatigue [3].

2.6. Secondary outcome measures

The secondary outcome measures included changes in health-related quality of life (HRQoL), sleep disturbances, anxiety, and depression through validated self-reported questionnaires.

2.6.1. The Short Form 36-item health survey

The 36-item short form health survey (SF-36) was used to assess health-related quality of life (HRQoL). The SF-36 is a broadly-based self-report survey on health-related physical and mental functioning status. It assesses functioning on eight subscales includ-

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ing domains of physical functioning, physical role, bodily pain, general health, social functioning, vitality, emotional role, and mental health, and two general subscales covering the physical and mental health domains on a 0–100 scale. Lower scores indicate a more negative impact of an individual's health on functioning [4].

2.6.2. Pittsburgh Sleep Quality Index

Sleep disturbances were assessed through the self-administered 19-item Pittsburgh Sleep Quality Index (PSQI) questionnaire. Scores are obtained on each of seven components of sleep quality: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep perturbations, use of sleeping medication, and daytime dysfunction. Each component is scored from 0 to 3 (0 = no sleep problems and 3 = severe sleep problems). The global PSQI score ranges from 0 to 21 points, with scores of ≥ 5 indicating poorer sleep quality [20].

2.6.3. Anxiety and Depression

Severity of symptoms of anxiety and depression were scored using the Hospital Anxiety and Depression Scale (HADS), a validated self-reported tool composed of 14 items (seven related to anxiety symptoms and seven to depression) among participants. Each item on the HADS questionnaire is scored from 0-3, and so scores range from 0 to 21; scores of 0-7 are interpreted as normal, 8-10 as mild, 11-14 as moderate and 15-21 as severe for either anxiety or depression. The total HADS score ranges from 0 (no anxiety or depression) to 42 (severe anxiety and depression) [5].

2.7. Circulating zinc levels

After overnight fasting for 12 hours, blood samples were collected from an antecubital vein using a vacutainer system (SST tubes, Becton Dickenson, Sarstedt, Barcelona, Spain) for each participant at baseline and each follow-up visit (at 8- and 16-weeks). Blood in SST tubes was allowed to clot at 25°C for approximately 15 min and was then centrifuged (Thermo Fisher Scientific, Heraus Megafuge 40, Langenselbold, Germany) at 1800x*g* for 10 min to obtain serum. Supernatants (serum samples) were pipetted into labeled 1.5 ml eppendorf tubes and stored at – 80°C until further analysis. Circulating zinc levels were assayed in each sample (100 µl) using an ESI SC-0400 One Fast system by inductively coupled plasma mass spectrometry (Thermo Scientific XSERIES 2 ICP-MS) at the Echevarne Laboratory (Barcelona, Spain). Each sample is analyzed together with five levels of calibrators, blanks and quality controls, all prepared with an amount of serum equivalent to that of the samples. The intra- and inter-assay coefficients of the variations were below 6%, and the low limit of detection was 80 µg/dL.

2.8. Sample size

Accepting an alpha risk of 5% and a beta risk of 20% in a two-sided paired test, 40 subjects were necessary in each group to obtain statistically significant differences, expected to be of 5% in the placebo group and 30% in the experimental group. A drop-out rate of 20% was anticipated (https://www.imim.es/ofertadeserveis/software-public/granmo/) [21].

2.9. Statistical analysis

The descriptive analysis was performed on the number of valid cases by means of absolute and relative frequencies, measures of central tendency (mean and median) and dispersion (standard deviations, SD and standard error of mean, SEM). Baseline demographic and clinical characteristic data were compared by *t*-Student test for continuous variables and by Fisher's exact test for categorical variables. Normality of the samples was verified by the Shapiro-Wilk test, and intragroup comparisons of continuous variables that followed a normal distribution were performed using the *t*-Student test for paired samples. If the hypothesis of normality could not be assumed, the analysis was carried out using the Wilcoxon test. For each intervention group, treatment effect was assessed as

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the mean change in variables from baseline to 8-, 16-weeks (score at 8- or 16-week visit minus score at baseline visit), and the treatment withdrawal effect was assessed as the mean change in variables from 16 weeks to four weeks' post-treatment (score at 4-week post-treatment visit minus score at 16-week visit). Between-treatment analysis was performed by Wilcoxon test at each visit. Data were analysed in an irreversibly anonymized fashion. Statistical analysis was carried out using the R-Studio desktop statistical software (version 1.3.1093). For all analyses, a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Demographic and clinical characteristics of the study population

Eighty eligible patients with ME/CFS were screened, of whom eight were excluded from the study because they did not meet the inclusion criteria. Of the 72 patients allocated and randomized in the study, 12 did not attend the baseline visit: nine (five in the experimental group and four in the placebo group) of their own accord and three (all from the experimental group) were considered lost due to limited mobility (spinal surgery, ankle sprain, and low back pain respectively). A total of 60 participants were visited at baseline, but only 50 (83%) completed the final assessment at 20 weeks; three participants (5%) were lost to follow-up (one in the placebo group with flares up of the disease, one in the experimental group with erythematous rash from taking ibuprofen, and another scheduled for hip surgery) and seven (12%) discontinued the study at their own request (five in the placebo group and two in the experimental group). The remaining ME/CFS patients completed all the study protocol procedures and were included in the analyses of outcome measures.

Baseline demographic and clinical characteristics of the study participants are presented in **Table 1**. No significant differences were observed in age, demographic and clinical data between the two groups at baseline. The placebo group consisted of 26 patients and the melatonin plus zinc group of 24 patients.

Table 1. Baseline demographic and clinical characteristics of study participants who completed the final assessment.

Characteristics	Placebo (n = 26)	Mel-Zinc (n = 24)	p-Value
Age (years)	53.7 ± 9.6	51.0 ± 10.2	0.339
Marital status			0.473
Married	15 (58)	16 (70)	
Single	7 (27)	6 (26)	
Separated/divorced	4 (15)	1 (4)	
Body mass index (kg/m^2)	28 ± 0.1	26 ± 0.3	0.651
Systolic blood pressure (mmHg)	118 ± 17.1	114 ± 13.7	0.291
Diastolic blood pressure (mmHg)	78 ± 11.9	72 ± 9.1	0.088
Heart rate (bpm)	75 ± 8	76 ± 12	0.680
Illness duration (years)			0.201
> 10	17 (65)	19 (79)	
≤ 10	6 (23)	4 (17)	
History of chronic pain	25 (96)	24 (100)	0.319
Illness-affected relatives	11 (42)	5 (21)	0.135
Concomitant drugs			
Anticonvulsants	13 (50)	18 (75)	0.217
Antidepressants	19 (73)	24 (85)	0.065
Anxiolytics	5 (19)	6 (25)	0.302
NSAID	12 (46)	11 (46)	0.720
Opioids	9 (34)	12 (50)	0.547

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Data are expressed as means ± standard deviations for continuous variables and compared by *t*-test, and categorical variables are given as numbers with percentages (%) and compared by Fisher's exact test. **Abbreviations**: Mel-Zinc, Melatonin plus Zinc; NSAID, Non-steroidal anti-inflammatory drugs.

3.2. Primary outcome measure

Table 2 shows the fatigue perception scores over the course of the clinical study in the two intervention groups. The perception of physical fatigue (assessed by the physical functioning domain) significantly improved at the 16-week visit in the melatonin plus zinc group (p = 0.026) compared to placebo group. Moreover, in the experimental group an improvement was observed from the beginning, which had reached statistical significance by the end of the treatment (16-week vs. baseline, p = 0.012), while in the placebo group the beneficial effect reached at eight weeks vs. baseline (p = 0.004) which was lost at the final treatment visit (**Figure 3A**).

Table 2. Fatigue severity (FIS-40 questionnaire) scores of the participants completing final assessment.

FIS-40 domains	Placebo (n = 26)	Mel-Zinc (n = 24)	p-Value[1]
Physical functioning			
Baseline	36.35 ± 4.74	36.29 ± 3.42	0.783
8 weeks	34.58 ± 4.12 *	35.38 ± 4.11	0.365
16 weeks	36.61 ± 4.44	34.25 ± 4.50 #	**0.026**
4 weeks post-treatment	36.29 ± 5.02	35.57 ± 3.65	0.151
Cognitive			
Baseline	35.08 ± 5.32	35.38 ± 5.59	0.930
8 weeks	34.04 ± 5.72	34.42 ± 5.65	0.907
16 weeks	34.61 ± 5.52	33.38 ± 6.24	0.584
4 weeks post-treatment	35.08 ± 6.58	35.13 ± 4.30	0.445
Psychological			
Baseline	64.88 ± 13.73	66.33 ± 9.98	0.930
8 weeks	63.46 ± 13.88	64.50 ± 9.64	0.861
16 weeks	66.61 ± 13.29	63.29 ± 10.22	0.150
4 weeks post-treatment	67.00 ± 16.76	65.98 ± 9.40	0.141
Total FIS-40 score			
Baseline	136.31 ± 21.93	138.00 ± 16.94	0.938
8 weeks	132.08 ± 22.65 †	134.29 ± 17.71	0.869
16 weeks	137.83 ± 22.23	130.92 ± 19.61	0.108
4 weeks post-treatment	138.38 ± 27.83	136.69 ± 15.42	0.139

Data are expressed as means ± standard deviations and compared by *t*-test where appropriate for intragroup analysis and by Wilcoxon test for intergroup analysis. * p = 0.004, 8-week values of the placebo group compared to baseline; # p = 0.012, 16-week values in the Mel-Zinc group compared to baseline. † p = 0.043, 8-week values of the placebo group compared to baseline. **Abbreviations:** Mel-Zinc, Melatonin plus Zinc; FIS-40, 40-item Fatigue Index Scale. Lower scores indicate an improvement in fatigue perception. [1] p-value for between-group analysis.

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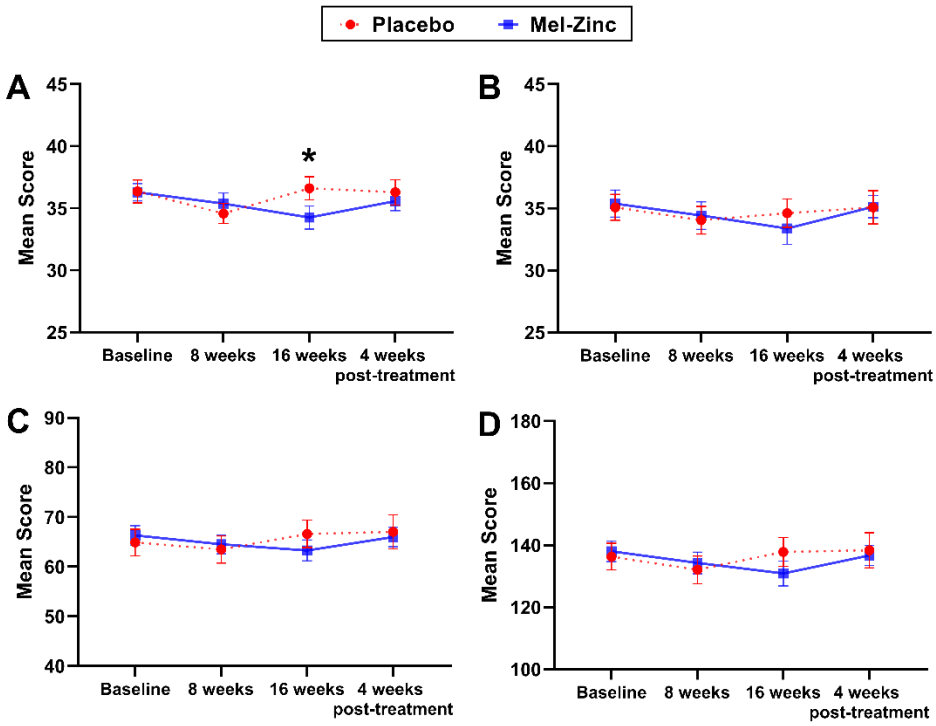

Figure 3. Changes in FIS-40 domain scores during the intervention study. Each point on the curve indicates mean FIS-40 item scores ± SEM. Lower scores indicate an improvement in the fatigue perception among participants. (**A**), Physical functioning domain; (**B**), Cognitive domain; (**C**), Psychological domain; (**D**), Total FIS-40 score. * $p = 0.026$, p-value for between-group analysis at 16-week visit by Wilcoxon test; no significant differences in any FIS-40 scores were observed between the intervention groups in the rest of study visits. **Abbreviations**: Mel-Zinc, Melatonin plus Zinc; FIS-40, 40-item Fatigue Index Scale.

As **Figure 3B** shows, with regard to the cognitive domain, both groups evolved in parallel; however, unlike the placebo group, the melatonin plus zinc group showed a trend towards improvement that was maintained over the 16 weeks of treatment, although it did not reach statistical significance (16-week vs. baseline, $p = 0.058$). The psychological domain evolved in the same way in both groups and over the course of treatment (**Figure 3C**). Finally, in the placebo group the FIS-40 total score showed an improvement ($p = 0.043$) at the 8-week visit with respect to baseline; this benefit disappeared at the 16-week visit, returning to baseline scores. In the melatonin plus zinc group there was a marked improvement at the 16-week visit, though without reaching significance (16-week vs. baseline, $p = 0.078$). The evolution of this parameter during the study did not differ between the groups (**Figure 3D**). Thus, a gradual improvement in all four domains was observed in the melatonin plus zinc group, which disappeared when treatment was withdrawn. In the placebo group, all four domains worsened at the 16-week visit while patients were still receiving treatment.

3.3. Secondary outcome measures

3.3.1. The Short Form 36-item Health Survey

Table 3 shows participants' scores for SF-36 questionnaire. In the melatonin plus zinc group, the physical functioning domain worsened significantly after treatment withdrawal (4-week post-treatment vs. 16-week, $p = 0.011$).

Table 3. Health-related quality of life (SF-36 questionnaire) in participants completing final assessment.

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SF-36 domains	Placebo (n = 26)	Mel-Zinc (n = 24)	*p*-Value [1]
Physical functioning			
Baseline	23.96 ± 19.22	22.92 ± 17.13	0.893
8 weeks	26.04 ± 20.75	21.04 ± 16.22	0.534
16 weeks	21.75 ± 19.21	26.09 ± 17.32	0.316
4 weeks post-treatment	26.14 ± 21.04	19.77 ± 16.44 *	0.421
Physical role functioning			
Baseline	0.00 ± 0.00	1.04 ± 5.10	0.298
8 weeks	6.00 ± 14.93	1.09 ± 5.21	0.179
16 weeks	3.41 ± 15.99	8.70 ± 25.68	0.323
4 weeks post-treatment	2.08 ± 7.06	4.35 ± 16.26	0.930
Bodily pain			
Baseline	14.35 ± 16.82	13.50 ± 13.75	0.848
8 weeks	20.08 ± 18.59 #	16.79 ± 12.61	0.797
16 weeks	17.35 ± 15.72	17.22 ± 17.05	0.847
4 weeks post-treatment	15.17 ± 16.66	13.78 ± 11.29	0.886
General health perception			
Baseline	14.50 ± 13.18	22.91 ± 11.13	0.015
8 weeks	21.23 ± 20.61 †	24.04 ± 14.90	0.197
16 weeks	15.55 ± 16.64	25.77 ± 16.47	0.017
4 weeks post-treatment	19.18 ± 16.47	22.05 ± 14.45	0.308
Vitality			
Baseline	18.59 ± 22.84	12.99 ± 14.17	0.678
8 weeks	23.40 ± 25.02	15.83 ± 19.04	0.370
16 weeks	16.01 ± 20.97	19.28 ± 23.26	0.482
4 weeks post-treatment	20.00 ± 23.95	18.64 ± 22.36	0.935
Social role functioning			
Baseline	32.69 ± 30.02	28.65 ± 17.86	0.835
8 weeks	41.35 ± 26.64 ‡	29.69 ± 19.44	0.154
16 weeks	31.52 ± 24.96	34.24 ± 20.72	0.488
4 weeks post-treatment	35.94 ± 32.41	32.07 ± 24.37	0.838
Emotional role functioning			
Baseline	46.15 ± 45.29	42.03 ± 44.06	0.790
8 weeks	41.03 ± 48.36	25.76 ± 38.40 §	0.403
16 weeks	46.97 ± 50.04	36.23 ± 43.71	0.507
4 weeks post-treatment	43.06 ± 49.62	28.99 ± 43.00	0.304
Mental health status			
Baseline	48.00 ± 21.32	47.63 ± 16.36	0.815
8 weeks	50.15 ± 24.66	45.13 ± 17.18	0.553
16 weeks	47.43 ± 20.72	50.43 ± 19.10	0.447
4 weeks post-treatment	47.91 ± 23.16	40.91 ± 18.92 ‖	0.368
Physical component summary			
Baseline	20.77 ± 5.71	22.53 ± 4.99	0.210
8 weeks	24.48 ± 6.50 ¦	24.25 ± 5.78 ◊	0.856
16 weeks	21.29 ± 7.89	25.12 ± 5.98 **	0.070
4 weeks post-treatment	22.85 ± 6.07	24.38 ± 6.03	0.335
Mental component summary			
Baseline	38.76 ± 13.32	35.94 ± 10.49	0.455
8 weeks	38.14 ± 14.25	33.61 ± 10.18	0.329
16 weeks	37.33 ± 14.14	35.95 ± 12.00	0.778
4 weeks post-treatment	38.52 ± 14.76	32.55 ± 12.73	0.201

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Data are expressed as means ± standard deviations and compared by *t*-test where appropriate for intragroup analysis, and by Wilcoxon test for intergroup analysis. * p= 0.011, 4-week post-treatment values of the Mel-Zinc group compared to 16-week values. # p= 0.020, 8-week values of the placebo group compared to baseline values. † p= 0.025, 8-week values of the placebo group compared to baseline values. ‡ p= 0.043, 8-week values of the placebo group compared to baseline values. § p= 0.022, 8-week values of the Mel-Zinc group compared to baseline values. ‖ p= 0.003, 4-week post-treatment values of the Mel-Zinc group compared to 16-week values. ¦ p= 0.001, 8-week values of the placebo group compared to baseline values. ◊ p= 0.023, 8-week values of the Mel-Zinc group compared to baseline values. ** p= 0.032, 16-week values of the Mel-Zinc group compared to baseline values. **Abbreviations:** Mel-Zinc, Melatonin plus Zinc; SF-36, 36-Item Short Form Health Survey. Higher scores indicate better quality of life. [1] p-values for between-group analysis.

The domains physical role functioning, bodily pain, general health perception, vitality, social role functioning, and emotional role functioning did not show relevant differences between the two groups. The mental health status domain evolved in a similar way without relevant changes. Only at the 4-week post-treatment visit was a significant worsening observed when melatonin plus zinc supplementation was discontinued (4-week post-treatment vs. 16-week, p = 0.003).

The physical component summary showed better scores in the placebo group at the 8-week visit, but this benefit was lost by the time of the 16-week visit. In the melatonin plus zinc group, statistically significant improvements were observed at both visits during treatment (8- and 16-week visits, p = 0.02 and p = 0.032 respectively). Between-group analysis did not show differences between the groups. In the mental component summary, no significant differences were observed either within or between the groups.

3.3.2. Pittsburgh Sleep Quality Index

Table 4 displays participants' sleep quality scores assessed using the PSQI. As in the primary endpoint, in the secondary endpoint PSQI scores in patients in the melatonin plus zinc group showed a progressive decrease that was maintained over the 16 weeks of treatment; in general, in the placebo group the evolution was more irregular, with a certain trend towards higher scores at 16 weeks compared to 8 weeks.

Table 4. Sleep quality (assessed with the PSQI questionnaire) in participants completing final assessment.

PSQI domains	Placebo (n = 26)	Mel-Zinc (n = 24)	p-Value [1]
Subjective sleep quality			
Baseline	2.46 ± 0.58	2.50 ± 0.66	0.692
8 weeks	1.92 ± 0.84 *	1.96 ± 1.00	0.791
16 weeks	1.96 ± 1.19 #	1.83 ± 1.01 †	0.540
4 weeks post-treatment	2.21 ± 0.78	1.91 ± 1.04	0.412
Sleep latency			
Baseline	2.46 ± 0.71	2.08 ± 0.83	0.096
8 weeks	1.88 ± 0.95 ‡	1.75 ± 0.90	0.589
16 weeks	2.00 ± 1.00 §	1.58 ± 0.93 †	0.128
4 weeks post-treatment	2.29 ± 0.91	1.83 ± 0.98 ‖	0.080
Sleep duration			
Baseline	1.92 ± 1.06	1.63 ± 1.21	0.366
8 weeks	1.54 ± 1.21 ¦	1.46 ± 1.14	0.817
16 weeks	1.70 ± 1.06	1.25 ± 1.26	0.172
4 weeks post-treatment	1.83 ± 1.15	1.91 ± 1.12 ◊	0.791
Habitual sleep efficiency			
Baseline	2.31 ± 0.93	1.83 ± 1.20	0.169
8 weeks	1.50 ± 1.24 **	1.75 ± 1.26	0.466

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16 weeks	1.91 ± 1.35	1.58 ± 1.41	0.524
4 weeks post-treatment	2.17 ± 1.07	2.04 ± 1.15	0.768
Sleep disturbances			
Baseline	2.31 ± 0.68	2.04 ± 0.55	0.110
8 weeks	1.92 ± 0.74 *	2.00 ± 0.51	0.642
16 weeks	2.09 ± 0.79	1.88 ± 0.61	0.312
4 weeks post-treatment	2.13 ± 0.85	2.22 ± 0.52 ##	0.825
Sleeping medication use			
Baseline	2.00 ± 1.30	1.83 ± 1.40	0.664
8 weeks	2.19 ± 1.30	1.58 ± 1.41	0.118
16 weeks	1.70 ± 1.40	1.38 ± 1.44	0.487
4 weeks post-treatment	2.25 ± 1.19	2.04 ± 1.36 ††	0.722
Daytime dysfunction			
Baseline	2.38 ± 0.85	2.58 ± 0.72	0.404
8 weeks	2.00 ± 0.85 §§	2.32 ± 0.76	0.158
16 weeks	2.13 ± 1.10	2.21 ± 0.93	0.991
4 weeks post-treatment	2.33 ± 0.76	2.48 ± 0.73	0.476
Global PSQI score			
Baseline	15.85 ± 3.21	14.50 ± 3.16	0.101
8 weeks	12.96 ± 4.51 ¦¦	12.83 ± 3.70 ◊◊	0.755
16 weeks	13.48 ± 5.33 ***	11.71 ± 4.72†††	0.178
4 weeks post-treatment	15.04 ± 4.70 §§§	14.43 ± 4.20 ◊	0.448

Data are expressed as means ± standard deviations and compared by *t*-test where appropriate for intragroup analysis and by Wilcoxon test for intergroup analysis. * $p = 0.008$, 8-week values of the placebo group compared to baseline values; # $p = 0.049$, 16-week values of the placebo group compared to baseline values. † $p = 0.005$, 16-week values of the Mel-Zinc group compared to baseline values. ‡ $p = 0.007$, 8-week values of the placebo group compared to baseline values. § $p = 0.002$, 16-week values of the placebo group compared to baseline values. ¦¦ $p = 0.041$, 4-week post-treatment values of the Mel-Zinc group compared to 16-week values. ¦ $p = 0.015$, 8-week values of the placebo group compared to baseline values. ◊ $p = 0.008$, 4-week post-treatment values of the Mel-Zinc group compared to 16-week values. ** $p = 0.000$, 8-week values of the placebo group compared to baseline values. ## $p = 0.006$, 4-week post-treatment values of the Mel-Zinc group compared to 16-week values. †† $p = 0.047$, 4-week post-treatment values of the Mel-Zinc group compared to 16-week values. §§ $p = 0.036$, 8-week values of the placebo group compared to baseline values. ¦¦ $p = 0.001$, 8-week values of the placebo group compared to baseline values. ◊◊ $p = 0.018$, 8-week values of the Mel-Zinc group compared to baseline values. *** $p = 0.006$, 16-week values of the placebo group compared to baseline values. ††† $p = 0.004$, 16-week values of the Mel-Zinc group compared to baseline values. §§§ $p = 0.037$, 4-week post-treatment values of the placebo group compared to 16-week values. **Abbreviations**: Mel-Zinc, Melatonin plus Zinc; PSQI, Pittsburgh Sleep Quality Index. Lower scores indicate an improvement in the sleep quality perception. [1] *p*-values for between-group analysis.

Sleep quality and sleep latency improved in both groups at the 16-week visit compared to baseline ($p = 0.005$ in both components for the experimental group, and $p = 0.049$ and $p = 0.002$, respectively, for the placebo group). In addition, in the melatonin plus zinc group an effect of treatment discontinuation for 30 days was observed, as sleep latency worsened ($p = 0.041$) compared to the 16-week visit.

In the melatonin plus zinc group, sleep duration, sleep disturbances, and sleep medication use worsened after 30 days of treatment withdrawal (4-week post-treatment vs. 16-week, $p = 0.008$, $p = 0.006$ and $p = 0.047$ respectively). On the other hand, in the placebo group, sleep duration, habitual sleep efficiency, sleep disturbances, and daytime dysfunction improved at the 8-week visit with respect to baseline ($p = 0.015$, $p = 0.0001$, $p = 0.008$ and $p = 0.036$ respectively), but this benefit was lost at the 16-week visit, returning to baseline levels.

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The global PSQI index showed significant improvements in both groups at the 8-week visit (p = 0.001 for the placebo group and p = 0.018 for the experimental group) and at 16-week visit (p = 0.006 for the placebo group and p = 0.004 for the experimental group). It also worsened after four weeks without treatment in both groups (p = 0.037 for the placebo group and p = 0.008 for the melatonin plus zinc group).

The between-group analysis did not show differences on any component of the self-administered scale.

Bedtime during study was between 23:45 ± 01:32 hours (earliest time average) and 24:00 ± 01:24 hours (latest average time) in the placebo group, and 23:13 ± 01:29 hours (earliest time average) and at 23:33 ± 01:13 hours (latest average time) in the melatonin plus zinc group.

3.3.3. Anxiety and Depression

Table 5 presents participants' anxiety and depression scores assessed through HADS. Anxiety improved at the 8-week visit in the placebo group (p = 0.004) although the scores had returned to baseline levels by the time of the 16-week visit. Paradoxically, there was an improvement after treatment withdrawal (4-week post-treatment vs. 16-week, p = 0.041). There were no changes in the melatonin plus zinc group.

Table 5. Anxiety and Depression scores (HADS questionnaire) of participants who completed the final assessment.

HADS domains	Placebo (n = 26)	Mel-Zinc (n = 24)	p-Value [1]
Anxiety			
Baseline	13.12 ± 4.74	11.92 ± 3.83	0.163
8 weeks	11.08 ± 4.49 *	12.46 ± 3.89	0.275
16 weeks	13.00 ± 5.29	11.67 ± 4.00	0.124
4 weeks post-treatment	12.50 ± 5.28 #	12.48 ± 3.65	0.543
Depression			
Baseline	11.62 ± 5.19	11.71 ± 3.37	0.718
8 weeks	10.77 ± 5.05	11.58 ± 4.30	0.907
16 weeks	11.87 ± 5.17	11.08 ± 4.05	0.353
4 weeks post-treatment	11.54 ± 4.81	11.74 ± 4.07	0.535
Total HADS			
Baseline	25.92 ± 6.90	21.92 ± 8.37	0.062
8 weeks	21.85 ± 9.05 †	24.04 ± 7.20	0.613
16 weeks	24.87 ± 9.94	22.75 ± 7.07	0.201
4 weeks post-treatment	24.04 ± 9.69	24.22 ± 6.69	0.400

Data are expressed as means ± standard deviations and compared by t-test where appropriate for intragroup analysis, and by Wilcoxon test for between-group analysis. * p = 0.004, 8-week values of the placebo group compared to baseline values. # p = 0.041, 4-week post-treatment values of the placebo group compared to 16-week values. † p = 0.028, 8-week values of the placebo group compared to baseline values. **Abbreviations**: Mel-Zinc, Melatonin plus Zinc; HADS, Hospital Anxiety and Depression Scale. Lower scores indicate an improvement in the severity of symptoms. [1] p-values for between-group analysis.

Depression showed no significant changes between visits in either group. The total HADS score improved at the 8-week visit in the placebo group (p=0.028) although it rose again to baseline values at the 16-week visit. In the melatonin plus zinc group there were no changes. The between-group analysis did not show differences in any of the domains of the self-administered scale.

3.3.4. Circulating zinc levels

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We further measured participants' serum zinc levels after melatonin plus zinc supplementation. No differences were observed in the evolution of zinc levels either over the course of the visits or between the groups (**Table 6**).

Table 6. Circulating zinc levels of the study participants who completed the final assessment.

Zinc, *μg/dl*	**Placebo (n = 26)**	**Mel-Zinc (n = 24)**	*p*-**Value**[1]
Baseline	114.73 ± 19.70	122.04 ± 31.68	0.676
8 weeks	114.42 ± 21.32	120.96 ± 24.58	0.336
16 weeks	126.62 ± 23.68	129.21 ± 31.40	0.641
4 weeks post-treatment	121.45 ± 19.71	127.87 ± 28.45	0.735

Data are expressed as means ± standard deviations and compared by *t*-test where appropriate for intragroup analysis, and by Wilcoxon test for between-group analysis. **Abbreviations**: Mel-Zinc, Melatonin plus Zinc; HADS. [1] *p*-values for between-group analysis.

3.3.5. Safety and tolerability

We report collective safety data for oral melatonin plus zinc supplementation study in patients with ME/CFS. Few adverse effects have been related to melatonin [21] and zinc [22]. In our study, no relevant treatment-related adverse events were recorded among study participants. These data demonstrate that the oral administration of melatonin plus zinc has a manageable safety and tolerability profile in people with ME/CFS.

4. Discussion

Research on the use of nutraceutical interventions as antioxidant supplements to reduce increased oxidative stress in individuals with ME/CFS remains controversial. Current evidence suggests that melatonin and zinc administration may achieve improvements in fatigue perception and health-related quality of life in these patients. In the present study, we failed to detect relevant improvements in sleep quality and anxiety, and depression. These results were unexpected, given that melatonin is the regulator of circadian rhythm, and zinc has been shown to be a useful treatment in anxiety/depression symptoms [15,24].

Several biochemical and immune abnormalities in inflammatory, oxidative and nitrosative stress pathways have been documented in ME/CFS, and recent studies have suggested that mitochondrial disturbances to energy requirements may be associated with its pathogenesis [7]. There is currently no treatment that modifies the natural evolution to chronicity, and so research efforts should focus on finding new molecules that improve the quality of life of these patients. As several nutritional deficiencies have been demonstrated in patients with ME/CFS, many attempts have been made to find therapeutic targets within natural nutritional supplements, although the results have been inconsistent [8,16].

The lack of a straightforward definition and clear-cut criteria for ME/CFS makes it difficult to compare the results of previous studies. The significant overlapping with other fatiguing diseases has caused major biases in many clinical trials for ME/CFS. Furthermore, since nutritional interventions have only recently been acknowledged as a valid part of a therapeutic approach, the body of evidence on their use is limited. Most of the trials conducted to date have been observational, and the interventional trials performed are not always of high quality; few well-conducted randomized controlled trials (RCT) have been performed in patients with ME/CFS. Another problem lies in the nature of nutritional supplementation, which may involve a single nutrient, or the combination of two nutrients with a synergistic biological function, or even cocktails of nutrients, making it even more difficult to analyze and draw conclusions [11]. Alterations in the levels of mel-

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atonin and zinc have been described in ME/CFS [25] but also in patients with fibromyalgia, a comorbid phenomenon very frequently associated with ME/CFS [26,27]. To our knowledge, this is the first randomized, placebo-controlled, double-blind trial to evaluate the potentially beneficial effects of oral melatonin plus zinc supplementation due to their potential synergistic antioxidant and anti-inflammatory effects. A previous study conducted by our group in people with ME/CFS evaluated the combination of CoQ10 and nicotinamide adenine dinucleotide (NADH), with a synergistic antioxidant effect, and showed an improvement in maximum heart rate and in the self-reported fatigue perception after an exercise challenge test (2-day CPET) [28]. Our results suggest that melatonin and zinc supplementation may have a positive effect on the perception of fatigue, as both intragroup (p = 0.012) and intergroup analyses (p = 0.026) showed statistically significant improvements in the physical dimension of the FIS-40 after 16 weeks of treatment (Figure 3A). Likewise, our nutrient combination may have influenced the cognitive dimension, with a progressive improvement at the end of treatment visit which almost reached statistical significance (Figure 3B, p = 0.058), and also the overall FIS-40 score (Figure 3D, p = 0.079). In view of the positive trend found for the melatonin plus zinc group, we consider that this study should be continued with a larger number of ME/CFS patients. In the experimental group, the physical functioning item worsened significantly after treatment withdrawal compared to the 16-week follow-up visit among participants (Table 3, p = 0.011).

Melatonin is a hormone produced by the pineal gland, predominantly at night, and carries out its action through membrane receptors (MT1, MT2) and nuclear receptors. These receptors are widely distributed in both the central and peripheral nervous systems and are associated with cellular differentiation and the regulation of the immune response [29]. They are also expressed on CD4+, CD8+ T cells and B-lymphocytes. Melatonin performs multiple functions in addition to its well-known role as the regulator of the circadian rhythm, such as regulating the redox system (eliminating free radicals that will lead to lipid peroxidation and acting on antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase), decreasing pro-inflammatory cytokines in the interests of immunomodulation, and acting as an anti-inflammatory agent by inhibiting macrophage/monocyte activation via the reduction of nitric oxide synthase activity [30]. Although the relationship between melatonin and fatigue has been studied in previous research, few studies have focused on ME/CFS.

In a study of melatonin in ME/CFS patients with delayed circadian rhythmicity, van Heukelom et al. [31] found significant improvements after treatment in fatigue, memory/concentration, motivation, and functional activity. Pardini et al. [32] conducted an efficacy study between melatonin and agomelatine (melatonin receptor agonist MT1 and MT2) in ME/CFS patients, reporting an improvement in the perception of fatigue in the agomelatine group. Starreveld et al. [33] found that light therapy improves chronic fatigue in (non-) Hodgkin lymphoma survivors through action on the suprachiasmatic nucleus, which stimulates the release of melatonin. Steur et al. [34] showed that the fatigue associated with acute lymphoblastic leukemia is related to changes in the sleep-wake rhythm. In addition to these studies, our group has recently demonstrated that ME/CFS patients present significant alterations in circadian rhythm patterns [12]. In our study, besides the benefit in physical fatigue, we observed a worsening in patient-reported PSQI parameters four weeks after withdrawal of treatment with melatonin plus zinc. Further studies with larger samples of ME/CFS patients determining the levels of melatonin and zinc during and after treatment are now warranted to confirm our findings.

Previous reports have suggested that dietary supplements such as CoQ10 and NADH are safe and well tolerated among ME/CFS patients, just as we found in our study with the combination of melatonin plus zinc. The most frequently reported adverse effects were daytime sleepiness, headache, and dizziness [22]. In fibromyalgia, an entity included within the central sensitization syndromes and ME/CFS-associated comorbid condition, several studies have been carried out with melatonin administration and have reported reductions in pain and improvements in sleep problems and healthy-related quality of life

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[35]. In multiple sclerosis, an entity with an immunoinflammatory basis in which fatigue plays an important role, the administration of melatonin has been also shown to improve fatigue and quality of life through the reduction of oxidative stress markers [36,37].

As regards zinc, decreased serum zinc concentrations have been identified as a biochemical marker of post-viral fatigue syndrome, depression, and activation of the virus-induced immune and inflammatory response [24]. Early clinical manifestations of lowered zinc status include fatigue, depression, and cognitive disorders [15]. Previous research has studied the relationship between zinc and chronic fatigue. Maes et al. [25] found a significant relationship between low zinc levels in serum and the values of the α-2 protein fraction and of T-cell activation markers in ME/CFS patients; this result could be explained by a reduction in the zinc levels caused by the sequestration of the intracellular heavy metal binding protein metallothionein in the liver, which, in turn, may be related to an increase in activity of IL-1β and IL-6; in a study of patients with colorectal cancer supplemented with zinc, De Figueiredo Ribeiro et al. [38] observed that fatigue was prevented and quality of life was maintained during chemotherapy. In our study, serum zinc levels in ME/CFS patients were within normal limits [24] and no differences were observed in the evolution of circulating zinc levels, either over the course of the visits or between the two groups.

Our literature search did not reveal any previous studies of supplementation with melatonin plus zinc in individuals with ME/CFS. However, it is known that the combined therapy of melatonin, vitamin C, plus zinc in other chronic diseases is more effective than the use of these agents alone, due to the synergistic effect of their action to modulate the oxidative stress and immune and inflammatory response (17). Patients with primary insomnia treated with melatonin, zinc, and magnesium supplementation, Rondanelli et al. [39] observed an improvement in sleep quality and in quality of life; also, a pre-clinical study in rats demonstrated that increased lipid peroxidation in muscle tissue due to ischemia-reperfusion may be prevented by melatonin and/or zinc supplementation [40].

The current study has several limitations that should be mentioned. Firstly, the sample size was relatively small (n = 50), and only women participated. While we applied a within-subjects design, future studies should seek larger samples and should explore sex-related differences. However, it should also be noted that recent studies have highlighted the utility of smaller samples with more robust measurement designs. Secondly derives from the recruitment site of the ME/CFS cohort. As all patients were recruited from a single tertiary referral center, the proportion of more severe patients may have been greater than normal, and so we should be cautious about generalizing the results to patients seen in other general healthcare settings (i.e., primary and/or outpatient care) or to the general population. Thirdly, doses and timing were pre-established, and so the dose-response effect cannot be analyzed. It might be useful to consider higher dosages (melatonin ≥ 2 mg at night and zinc: 30-50 mg/day) and longer interventions (more than 6-12 months) in order to determine their potential benefit. Fourthly, for logistical reasons, urinary melatonin levels were not measured for each participant. Finally, we did not control for confounding factors such as diets, habits and lifestyles among participants. We have no reason to believe that their daily diets and/or lifestyle changed between sessions, but future prospective cohort studies should employ dietary habits reports or other accounts to examine these important lifestyle factors in ME/CFS population.

Our study also has several important strengths. First, it is the first pilot study to assess the effect of oral melatonin plus zinc supplementation in ME/CFS. Second, the combination of melatonin plus zinc improved the perception of physical fatigue and physical quality of life in ME/CFS patients. Third, the withdrawal of the nutritional supplement had a deleterious effect on the sleep quality and health-related quality of life in the study population. Fourth, the use of strict inclusion criteria based on the 1994 CDC/Fukuda case definition for ME/CFS ensured that the participants were appropriately selected and did not have confounding psychiatric comorbidities. Fifth, the combination of melatonin plus zinc was safe and well tolerated among participants. Larger multicenter trials with longer follow-up interventions in more homogeneous ME/CFS populations, examining not only

melatonin and zinc levels but also immune and inflammatory response biological markers and the redox system, are now warranted to assess these findings and to produce evidence-based guidelines regarding the potential beneficial effects of antioxidant therapy in ME/CFS and in other fatiguing chronic conditions.

5. Conclusions

To the best of our knowledge, this is the first pilot study to assess the effect of melatonin plus zinc supplementation in individuals with ME/CFS. We found that oral melatonin plus zinc supplementation significantly improved the perception of physical fatigue and health-related quality of life in ME/CFS patients after 16 weeks of treatment. A treatment withdrawal effect was observed in the melatonin plus zinc group, with symptomatic relapse in the sleep parameters and in the physical function and mental health dimensions (SF-36), but not in the placebo group. Based on these results, the administration of melatonin (1 mg) and zinc (10 mg) daily may be indicated as adjuvant treatment for ME/CFS patients to improve their fatigue. Additional well-powered intervention studies should be conducted to attain a full assessment of the antioxidant and anti-inflammatory effects of oral melatonin and zinc supplementation on core symptoms and biological mechanisms in people with ME/CFS.

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Institutional Review Board Statement: All study procedures were reviewed and approved by the local Clinical Research Ethics Committee of the Vall d'Hebron University Hospital, Barcelona, Spain (reference code: IC/LV/MEL-ZN/SFC, approved on February 16, 2016). The study was conducted according to the guidelines of the Declaration of Helsinki, and with the current Spanish regulations on clinical research and the standards of Good Clinical Practice of the European Union. It also followed the Consolidated Standards of Reporting Trials (CONSORT) guidelines.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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