

Clinical improvement in chronic fatigue syndrome is associated with enhanced natural killer cell-mediated cytotoxicity: the results of a pilot study with Isoprinosine[®]

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Abstract. *Chronic fatigue syndrome is associated with systemic and cognitive symptoms and with several immune abnormalities. The clinical impact of Isoprinosine[®] was evaluated in sixteen CFS patients, followed for 28 weeks in a single-blind, placebo controlled trial. Patients were also monitored for various immune parameters. Improvement based on clinical staging was observed in six of ten treated patients (60%). Clinically improved patients showed significantly enhanced natural killer (NK) cell activity, which correlated with the duration of Isoprinosine[®] treatment ($p < 0.03$). Treatment with Isoprinosine[®] resulted in significantly increased numbers of CD4+ T helper cells ($p < 0.03$). Treatment with Isoprinosine[®] for 12 weeks did not appreciably influence the in vitro production of IFN- γ , IL-1 α , IL-10 or IL-12. However, IL-12 was significantly increased at week 28 ($p < 0.02$) in patients who improved after treatment with Isoprinosine[®]. These results suggest that taking Isoprinosine[®] may benefit a subgroup of patients with CFS, and this clinical improvement is associated with enhanced NK cell function and IL-12 levels. Further trials to evaluate the use of Isoprinosine[®] in the treatment of CFS patients are warranted.*

Keywords: chronic fatigue syndrome, Isoprinosine[®], IFN- γ , IL-12, IL-10, IL-2, Natural Killer Cells

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INTRODUCTION.

The 1988 and 1994 Center for Disease Control criteria for chronic fatigue syndrome (CFS), include a requirement for several symptoms, such as severe fatigue, exercise intolerance, myalgia, cognitive deficit and a variety of neuropsychological symptoms (1,2). Many studies suggest the involvement of the immune system in the pathogenesis of CFS (3-8). Unfortunately, there is no established treatment for CFS, although several therapies, including a variety of immunomodulatory

therapies, have been attempted (9-17).

Isoprinosine[®] (inosine pranobex) is a synthetic purine derivative consisting of the β acetamidobenzoic acid salt of N, N-dimethyl-amino-2-propanol (DIP.PAcBA) and β polymorph of inosine in a 3:1 molar ratio. It is an immunopharmacologic agent with both immunomodulatory and antiviral properties. Isoprinosine[®] has been licensed since 1971 for the treatment of cell mediated immune deficiencies associated with various viral infections (18-20). The exact mechanism for these actions is not fully understood. However,

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Isoprinosine® has been shown to modulate CD4+ and CD8+ T cell functions (21-23), stimulate NK cell activity *in vitro* and *in vivo* (24-27), and normalise a deficient or dysfunctional cell-mediated immunity by evoking a TH1-type response (28,29). Isoprinosine® also increases the production of cytokines such as IL-1 α , IL-2, and IFN- γ while decreasing IL-4 production (30-32). Safety data from clinical trials (33-36) and many years of cumulative adverse drug event post-marketing experience demonstrated the safety of Isoprinosine® on large numbers of patients during short and long term administration.

Since CFS is associated with a variety of immune abnormalities including suppression of NK cell activity and altered production of cytokines such as IL-4 (37), IL-6 (38) and IL-12 (our unpublished observation), we hypothesized that Isoprinosine® administration may enhance NK cell activity and IL-12 production and may have the potential to influence the clinical course of CFS. Therefore, this study investigated how a subgroup of CFS patients would benefit from the administration of Isoprinosine®. We analyzed the safety of Isoprinosine® as well as the correlation between immune dysfunction and clinical improvement.

METHODS.

Selection of patients and baseline characteristics.

Sixteen Caucasian patients, 13 women and 3 men, were referred from the Nightingale Research Foundation in Ottawa. At baseline, the mean age of the patients was 45 years 8 months (SD 8 years 4 months). Fourteen patients had a diagnosis of chronic fatigue syndrome as defined in the 1988 and 1994 CDC revised case definition for CFS. Two patients also had a diagnosis of depression. All patients had ongoing symptoms for six months or longer and were aged eighteen to sixty years. Females of childbearing potential had a negative standard HCG pregnancy test. The study was conducted at Cytos Clinical Research in Ottawa, and the laboratory analysis was performed at the Children's Hospital of Eastern Ontario (CHEO), also in Ottawa. An independent ethics

committee approved the study and all patients signed a consent form before enrolment in the study. Prior to the study, all sixteen patients underwent a diagnostic work-up to exclude malignancy and major organ or system pathology inconsistent with CFS. Eight of the thirteen women in the study had significant signs of fibromyalgia (FMS), but none of the men showed evidence of this syndrome. Monthly physical examinations and blood and urine tests were performed on each of the sixteen patients during the trial; no serious adverse events were reported. In some cases, transient elevation of serum uric acid levels (usually returning to baseline values a few days after the end of treatment) was observed. This undesirable effect was possibly due to metabolism of the inosine moiety through the normal purine metabolic pathway. Immune studies were performed on all patients prior to and during the course of the investigation; these included cytokine analysis and CD4+, CD8+ T cell, B cell, and NK cell level determination by flow cytometry.

Patients were allowed to continue their pre-existing medications with the exception of those prohibited by the protocol. No patient was on immunomodulatory drugs, antivirals, investigational drugs, pharmacological agents that interfere with nucleic acid biosynthesis, xanthine oxidase inhibitors, Vitamin C supplements or herbal medicines. Concomitant medications during the blind-phase were as follows: 5 patients were taking hypnotics for the treatment of insomnia, 2 patients were on anticonvulsants, 3 were on antidepressants, 3 were on anxiolytics, 4 on anti-allergic drugs, and 5 were on proton pump inhibitors or H₂ receptor antagonists. Seven patients were taking non-steroidal anti-inflammatory drugs, 2 stopped taking their medications during the trial.

Study design

Dosing and duration of treatment

Patients were randomly assigned to receive either a 500 mg inosine pranobex (Isoprinosine®) tablet (n=10) or a methylcellulose placebo tablet (n=6) according to the following cyclic dosing schedule:

-Weeks 1, 3, 5, 7, 9, and 11 inclusive, Monday through Friday only: two tablets orally, three times daily (6x500 mg, i.e. 3 g/day) during waking hours

-Weeks 2, 4, 6, 8, 10, and 12 inclusive, Monday through Friday only: two tablets orally, (2x500 mg, i.e. 1 g/day), in the morning

Blinding

In this randomised, single-blind study, ten patients were given Isoprinosine® for three months while six patients were given a placebo. At the end of the three month period (blind phase: weeks 0-12), both the placebo and the Isoprinosine® group ceased all medication for a one month period. All sixteen patients were informed as to whether they had been on placebo or Isoprinosine®. Both groups were then given the choice of continuing the medication for another three months (open-label phase: weeks 16-28).

Compliance

One of the female patients from the placebo group dropped out of the blind phase. Another female patient in the placebo group dropped out of the open-label phase. Neither dropout was due to adverse effects. The blind phase dropout was omitted from the intention to treat analysis because the subject had not begun the medication schedule.

Measures of outcome.

Questionnaire assessments.

There is no diagnostic test or pattern of tests that can assist in the diagnosis of CFS. Rather, CFS is diagnosed on the basis of subjective complaints and exclusion criteria. Many of the trials report immune function or other laboratory measures as outcomes, but the only outcome that is likely to matter to patients is how they feel (39). Because there are frequent inconsistencies in observed immune function changes, it may not be appropriate to use immune measures alone as clinical markers for CFS.

We chose the following three

standardised tests to follow up our patients: the Activities of Daily Living Questionnaire (ADL), the Cognitive Deficit Subset of the Symptom Checklist Questionnaire (SCL-90-R), and the Karnofsky Performance Score (KPS). Test scores were recorded every four weeks from baseline. All of these outcomes rely on patients' self-reports of their symptoms and levels of activity.

Cognitive deficit subset of the Symptom Checklist Questionnaire (SCL-90-R)

Subjective complaints of perceived cognitive deficits in CFS patients were described by Freidberg *et al.* (40), and impairment in objective cognitive performance was reported by DeLuca *et al.* (41), Christodoulou *et al.* (42), and Lawrie *et al.* (43). Therefore, we included the SCL-90-R questionnaire in this study because it is a valid, psychometric, self-assessment instrument (44,45). Eight cognitive assessment questions are buried within a series of ninety other questions, which are listed in Appendix A. A score of 4 reflects extreme impairment and 0 reflects no impairment. The SCL-90-R cognitive deficit score for each patient was calculated as the mean score for the eight cognitive subset items. Higher values represent greater perceived cognitive deficit.

Levin *et al.* found significant differences in the individual and summary scores for the Global Severity Index (GSI) profile of the SCL-90-R between fatigued patients and healthy controls (46); therefore, we also calculated the GSI for each patient. The GSI is based on the mean of all the SCL-90-R scores. Each subject's test scores were transformed to a standard score by comparing their raw scores to normative values.

Activities of Daily Living (ADL) scores

Numerous studies suggest that fatigue has a strong association with functional impairment and compromises the activities of daily living (47-50). Several methods of rating fatigue and extent of physical activity have been described using performance-based measurements and/or self-reports (51-56). It is of great sociocultural importance to have

information about independence of self-care needs in a therapeutic trial. Thus, we included a self-administered questionnaire about activities routinely encountered in daily living.

ADL was evaluated by using the modified Barthel Index that consists of eighty-three items from thirteen categories of daily activities (57-60). Five different levels of performance measure the thirteen modules in the ADL. The maximum score for a specific task is 100 (no symptoms, no help needed); the lowest score is 20 (unable to do).

There were too few patients in each group to permit factor analysis as a means of measuring overall results of the Activities of Daily Living questionnaire. Consequently, we calculated the overall ADL scores by averaging the scores in thirteen modules then multiplying the mean by 20.

Karnofsky performance score (KPS)

The Karnofsky performance scale is a modified questionnaire originally developed for rating the QoL of patients undergoing chemotherapy (61). The scale has been used as an outcome measure in several controlled therapeutic trials on CFS patients (11,16,17). A change of status was indicated if the KPS result differed by at least ten points. Higher scores indicate clinical improvement.

Fibromyalgia

It has been estimated that 35% to 70% of those with CFS also have fibromyalgia, and they share key symptoms such as muscle pain, aches, sleep disturbances, and difficulty concentrating (62-66). Tender points, one of the ACR diagnostic criteria for FMS (67), are also common in CFS (68). In addition to the outcome measures, therefore, at each visit our patients were tested for the number of tender points they experienced.

Immunological assessments

A series of immunological tests were performed on the sixteen subjects: determination of CD4+ and CD8+ T cell numbers, CD4/CD8+ T cell ratio, HLA-DR+CD4+ T cell numbers,

CD16/CD56+ NK cell numbers, and NK cell activity.

Peripheral blood lymphocytes from CFS patients were analyzed for NK cell activity against K562 target cells. K562 cells (1×10^4) were labeled with ^{51}Cr and incubated for 4 hrs with PBMC from CFS patients at effector-target cell ratios ranging from 1:2 to 1: 100. Specific cell lysis was measured by ^{51}Cr release in a γ counter. The specific lysis was calculated as follows:

$$\% \text{ Specific Lysis } \times 100 =$$

$$\frac{X \text{ experimental cpm release} - X \text{ spontaneous cpm release}}{X \text{ maximal cpm release} - X \text{ spontaneous cpm release}}$$

Specific cell lysis was represented as lytic units (LU). LU-5% per 10^6 effector cells. One lytic unit is defined as the number of effector cells needed to lyse 5 % of the 10×10^3 target cells. Similarly, one LU-15 % is defined as the number of effector cells needed to lyse 15 % of the 10×10^3 target cells.

Cell stimulation and collection of culture supernatants

PBMC (2×10^6 cells/ml) from CFS patients were stimulated with PHA (1:50 final dilution), anti-CD3 (Anti-CD3 antibody-secreting hybridoma, OKT 3, obtained from American Type Culture Collection, ATCC, Rockville, MD; 1:200 final dilution of the culture supernatant), LPS (Sigma, Oakville, ON; 1:100 final dilution) or PWM (Sigma, Oakville, ON; 1:100 final dilution) in a 24-well tissue culture plate (Falcon, Becton Dickinson, Lincoln Park, NJ). The supernatants were harvested after 48 hours, centrifuged for 10 minutes at 1600 rpm, transferred to a fresh tube and frozen at -70°C . Supernatants were thawed at the time of analysis for measurement of IL-12 and IFN- γ cytokine production by enzyme linked immunosorbent assay (ELISA).

Measurements of Cytokines produced by T cell mitogen activated PBMC

Cytokine production, including IL-1 α , IL-2, IL-10, IL-12, and IFN- γ were measured in the culture supernatant of PBMC after T cell

mitogen activation with either PHA or anti CD3 antibodies. Cytokines were measured by ELISA using two different monoclonal antibodies (Mab) which recognize distinct epitopes. Briefly, 96-well plates (Nunc Immunomodules, Roskilde, Denmark) were coated overnight at 4° C with the primary antibody at a concentration of 4 µg/ml in coating buffer (0.1 M NaHCO₃, pH 8.2). The plates were washed six times with PBS-Tween 20 and blocked with PBS/10% FBS for 4 hours. Recombinant cytokine standards and sample supernatants were diluted in IMDM added to the plates and incubated at 4° C overnight. The wash step was repeated. The cytokines were detected by incubating for 2 hours with a second biotinylated Mab in PBS/10%. All monoclonal antibodies and reagents for the ELISAs were obtained from Medicorp, Montreal, Quebec. After another wash step, streptavidin-peroxidase was used at a final dilution of 1:1000 to detect cytokine levels; plates were incubated for a further 2 hours. The color reaction was developed by OPD and hydrogen peroxide addition to the plate for 30 minutes. The reaction was stopped with 0.1 M HCl and the plate was read at 490 nm. The results are expressed as pg/ml for all the cytokines.

Blood samples were obtained on the same days that the questionnaire assessments were performed (at baseline and at the end of weeks 12, 16, and 28). Peripheral blood was collected between the hours of 8:00 am and 12:00 PM. Lymphocytes were isolated in culture within 4 hours of sample collection. All of the laboratory measurements were conducted without the knowledge of the diagnosis or the randomisation code. Immunological data were correlated with clinical status only after the clinical results had been tabulated.

Statistical methods

The main analysis (blind phase, week 0-12) included comparisons of the stratified groups using non-parametric equivalents to the standard t test (Kruskal-Wallis test, Wilcoxon rank sum test). The tests were two-tailed with a α -level of 5%. Results at weeks 12, 16 and 28 were compared with week 0. Open-label phase results are not detailed in this paper, except for

data on IL-12 and CD4 T cells expressing HLA-DR.

Clinical classifications

Clinical staging was used to classify patients into three clinical subgroups, determined independently from the primary outcome measures and carried out prior to analyses of the outcome measures at the end of the trial. The stratification was based on clinical staging that consisted of the Patients' self-report to the investigator and the results of an Investigator Assessment Form (IAF) (see Appendix B). The study-specific IAF was used to assess patients' symptoms during each visit and to ensure more consistency in the parameters recorded. The checklist included symptoms derived from published criteria for CFS (1,2) in addition to well-recognised features used in clinical diagnosis. In the IAF, the CFS symptom severity measure was based on an additive score of twenty symptoms. The responses were coded using a four-point scale ranging from 0-3 (0=absent, 3=severe).

At the end of the blind phase (week 12), patients were classified as treated Improved, treated Not Improved, or Placebo. The Improved group (4 females and 2 males) consisted of CFS patients who received active trial medication between week 0-12, and reported substantial improvement in their symptoms (Patients' self-report to investigator) for at least two months during the blind phase. All but one of the patients who reported significant improvement to the investigator experienced a decrease in their mean IAF symptom severity score of at least 25%. The Not-Improved group (4 females) received active trial medication between week 0-12, but continued to experience pre-study symptoms and reduced level of physical activity (Patients' self-report to investigator) and showed a decrease of less than 25% on their IAF symptom severity scores. The Placebo group (4 females and 1 male) received placebo trial medication between week 0-12.

Table 1 details the clinical classification based on the IAF data and the Investigator's summary of the patients' self-reports for the Improved and Not-Improved groups. There was

a strong correlation between the investigator's evaluation of the patients' self-reports and the IAF scores.

Based on the patients' self-reports to the Investigator, clinical improvement was observed in 6 out of 10 patients in the treatment arm at the end of the blind phase. None of the Placebo patients self-reported improvements in their overall well-being, even though their IAF scores showed some improvement. At the end of the blind phase (week 12) the Improved group symptom severity score decreased 32.9% compared to decreases of 8.8% and 21.6% for the Not-Improved and the Placebo groups, respectively.

RESULTS

Among the numerous studies on CFS, very few discuss the results of detailed immunological investigations of drug therapy and compare them with clinical/neuropsychological outcome measures. Therefore in our trial, we first report the findings of the three standardised questionnaires then we describe the results measuring various immune functions.

Questionnaire Assessments

Results of the cognitive deficit subset of the SCL-90-R are summarised in Table 2. At baseline, both the Improved and the Placebo group showed perceived cognitive deficit, while the Not-Improved group scored close to the upper limit but in the normal range. At the end of the blind and open-label phases (data not shown), the median percentage reduction in cognitive symptoms was largest for the clinically improved group (-16%). None of the differences between groups, however, was statistically significant (Kruskal-Wallis $p=0.43$). The test of the Global Severity Index (GSI) score changes of week 12 from baseline indicated no statistically significant difference between the three groups (Kruskal-Wallis $p=0.75$).

No statistically significant differences between the three groups were detected for the Activities of Daily Living questionnaire.

On the Karnofsky Performance scale, two patients' scores from the Improved group

increased by 17% from week 12 over baseline (Table 3). The KPS results of the other four Improved subjects remained unchanged. Among the Not-Improved group, one of the four patients worsened, one showed improvement, and the others were unchanged. Test results remained unchanged for all five patients on placebo.

Fibromyalgia

The results of the tender point tests in our study support the findings of previous studies concerning the considerable overlap of symptoms in FMS and CFS (69-71). Both of the Treated (Improved and Not-Improved) groups exhibited a median decrease in tender points at the end of the blind phase from 12.5 to 10 and 10 to 8.5, respectively (Table 4). Meanwhile, the Placebo group median increased by 1 tender point. The differences between groups for the changes above baseline, however, were not statistically significant. The greatest decreases in tender points were observed at the end of the open phase (week 28). The clinically Improved group exhibited a statistically significant median decrease of 8 tender points (Kruskal-Wallis $p<0.03$), compared with increases of 2 and 0.5 for Not-Improved and Control groups, respectively (data not shown).

Measurement of Immune Functions

Clinical improvement in patients was associated with an increase in NK cell activity (Tables 5 and 6). There was a significant increase in the NK lytic activity in the Improved patients vs. Not-Improved (23.7 ± 7.5 vs. 14.7 ± 3.4 , respectively, Kruskal-Wallis $p < 0.03$). Both the Improved and the Not-Improved subgroups of the treatment arm also showed increased NK cell numbers at week 12, although the changes were not statistically significant (Wilcoxon $p = 0.09$). Discontinuation of treatment resulted in a reduction of median NK cell activity, from 23.7 LU at week 12 to 12.8 LU at week 16 in the Improved group, to 14.7 LU at week 12 to 10.1 LU at week 16 or four weeks after discontinuation of Isoprinosine®. Reinitiating treatment caused an increase in NK cell activity in the open phase of the study (data

not shown).

Treatment with Isoprinosine® resulted in significantly greater numbers of CD4+ T helper cells in the Improved Group at Week 12 (Kruskal-Wallis test for difference between groups at week 12 $p < 0.03$, Table 7 and Figure 1; medians by group are: Improved 995.5, Not-Improved 659.5, Placebo 575). Although clinically improved patients showed enhanced expression of the activation marker HLA-DR+ in CD4+ T cells at week 12, the differences between groups were not significant ($p = 0.19$). However, treatment with Isoprinosine® for 28 weeks resulted in an increased number of CD4+ HLA-DR+ T cells (Figure 2; Kruskal-Wallis test for difference between groups at week 28 $p < 0.05$ (medians [IQRs] by group are: Improved 420 [303, 550], Not-Improved 182 [174, 304], Control 257 [183.5, 312.5]).

We also measured the ability of patients' lymphocytes to produce cytokines that are associated with inflammatory reaction (IL-1 α), cytokines produced by NK cells (IL-12), cytokines implicated in the development of T helper responses (IL-10, IL-12 and IFN- γ), and cytokines produced by monocytes/ macrophages (IL-10, IL-12). Results are depicted in Table 7. Treatment with Isoprinosine® for 12 weeks did not appreciably influence the *in vitro* production of IL-1 α , IL-10, IL-12 or IFN- γ . The Improved group at week 12 did show higher median levels of IL-2; however, the difference between groups was not statistically significant ($p = 0.21$).

Treatment with Isoprinosine® for 28 weeks resulted in enhanced production of IL-12 in the Improved group. IL-12 increased from a median of 50±66 pg/ml before Isoprinosine® treatment to a median concentration of 152±49 pg/ml at week 28 in anti-cd3 stimulated PBMCs (Wilcoxon $p < 0.02$). In comparison, there was no statistically significant change in IL-12 between week 0 and week 28 in the Not-Improved or Control (placebo) groups. At week 28, IL-12 in the Improved group (median 152±49) was also higher than IL-12 in the Not-Improved group (median 72±53), but the difference was not statistically significant (Kruskal-Wallis $p < 0.07$).

DISCUSSION

Among the variety of immunological abnormalities that have been reported, numerous conflicting claims have been published about deficits in the absolute numbers of natural killer (NK) cells (72-74). According to reports by Barker *et al.*, Behan *et al.*, Caligiury *et al.*, Kibler *et al.*, Klimas *et al.*, Masuda *et al.*, Morrison *et al.*, Patarca *et al.*, and Whiteside *et al.*, there is more consensus about the impairment of functional activity of NK cells in CFS (75-83). Except for the findings of Gold *et al.*, decreased NK cell activity is one of the most consistent immunological observations (84). Similarly, decreased NK cell activity was also shown in fibromyalgia syndrome (FMS), a condition that shares many symptoms with CFS (85-86). Several published experiences of *in vitro* and *in vivo* studies discuss the positive effect of Isoprinosine® on NK cell activity (22,24,26,27). Likewise, results from this study showed statistical evidence that reduction of symptom severity and improvement in daily physical function is associated with increased NK cell activity. Results of the study by Cruess *et al.* suggest that the degree of cellular immune activation is associated with the severity of CFS-related physical symptoms, cognitive complaints, and perceived impairment secondary to CFS (87). Namely, the decreased percentage of CD3+CD8+ cells and the increased number of CD38+HLA-DR+CD8+ cells were the strongest predictors of total illness burden. Hilgers and Frank found significant positive correlations between the 30-criteria-score and the numbers of CD8+ T-cells, HLA-DR+ T-cells, gamma-globulins, IgG, IgM, and autoantibodies (88). A significant reduction in CD8+ T-cell numbers and an increase in activation markers (CD38+, HLA-DR+) on CD8+ cells were found by Landay *et al.* in patients with CFS (5). Moreover, in two cases, some immunological markers returned to normal with an improvement in symptoms. Unlike Peakman *et al.*, where clinical improvement in CFS was not found to be associated with lymphocyte subsets of function or activation, we found an association between clinical improvement and NK cell activity (74).

After a four-week treatment-free period at the end of the blind phase, five of the six Improved patients and all of the Placebo patients

decided to participate in the open-label phase for a further 12 weeks of therapy. Because of these changes, subjects in the active treatment groups had different durations of exposure to Isoprinosine® compared to subjects in the Placebo group. After the blind phase, five of the six Improved patients had additional 12 weeks of therapy, (a total of 24 weeks), while the patients who were on placebo during the blind phase were treated for only 12 weeks during the open-label phase. Because these differences might have implications for treatment efficacy and consequently for statistical analysis, we tended to focus on changes that occurred between baseline and week 12 (end of blind phase) rather than between baseline and week 28 (end of open-label phase). It is of interest that none of the Placebo patient self-reported improvements in their overall well being at the end of the blind phase even though the standardised tests showed some improvement.

In view of our results showing an increase in IL-12 production and CD4+ HLA-DR+ cells after 28 weeks of treatment with Isoprinosine®, we hypothesize that improvement in several immune function assays may require an extended treatment with this drug. Therefore, it is likely that a strong correlation between NK cell activity and NK cell number in clinically improved patients may be observed over a long period of treatment and it may be related to an increase in IL-12 production.

Despite the trends for improvement in the SCL-90-R and Karnofsky test results for the Improved group, the differences between groups were not statistically significant. The overall lack of statistically significant differences between groups after the therapy raises several issues. First, the small sample size made it difficult to achieve statistical significance on tests, particularly given the stratification that limited the largest group to six subjects. Furthermore, the restricted sample size did not permit us to correct for covariates such as gender, duration of disease, and level of functional impairment. Second, it calls into question whether the cognitive deficit subscale of the SCL-90-R or its overall GSI are sensitive enough to detect moderate neuropsychological changes in response to drug therapy in CFS

patients. In addition, from the baseline GSI scores, eight of the sixteen diagnosed CFS patients did not qualify as clinical cases (scored less than or equal to the cut-off score of 63). Third, the analysis of the ADL results was limited because the frequency of testing did not provide enough information to reliably track trends in functional changes following the therapy. More frequent collection of ADL data or continuous monitoring of functional levels may be useful in future therapeutic trials. Finally, one can speculate that the sampling restriction may be a reason why KPS was not a sensitive indicator of change in this cohort. Considering the length of the trial and the frequency of the follow up visits, one of the enrolment criteria was that patients score at least 60 points on the KPS in order to ensure subject compliance. Only those patients who were likely to tolerate the study procedure were included (i.e. those who could walk without assistance). In summary, these results may indicate that the chosen standardised tests were not particularly suitable to our subgroup of CFS patients for follow-up of clinical changes during drug therapy.

Given that our results demonstrate an association between clinical improvement and enhanced NK cell activity and increases in CD4+ T helper cells, CD4+ HLA-DR+ cells, and IL-12 in a subset of CFS patients, analysis of immune function may be a surrogate marker of disease severity and therefore an indicator of therapeutic efficacy.

Although the number of patients was too small to lead to firm conclusions, the temporal relationship between therapy and recovery accompanied by the significant association with increased NK cell activity raised the possibility of treatment-mediated response. Following immunomodulatory therapy, however, none of the immunological changes could be directly associated with clinical symptoms. A possible explanation is that the chronic existing immune activation and consequent decrease in NK cell activity could be restored with immunomodulator therapy. These conclusions add important additional information to the concept of how subtle immunological abnormalities change during therapy. In addition to patients' self-reports, immunological

testing could be an important indicator for follow-up drug therapy in CFS. Therefore, the results of this pilot study are valuable since clinical indicators in response to CFS drug therapy are still poorly understood.

The present study has several limitations; the most prominent problem in clinical trials with CFS is that the clinical changes still have to rely on patients' self-assessment reports following drug therapy. As well, due to the lack of consistent immunobiological markers and physical findings during diagnosis, the precise definition of improvement is difficult at this stage.

In order to draw firmer conclusions, this pilot study should be followed up by a Phase IIb/Phase III trial using an adequate sample size. This would lead to a greater understanding of the correlation between fatigue, decreased daily activity, and reduction in number and activity of NK cells. Therefore, the application of a standard fatigue scale or a performance-based measurement with parallel testing of these immunological markers is strongly recommended.

Finally, given the wide fluctuation of symptoms and the tendency for spontaneous recovery during the long duration of CFS, a longer follow-up (≥ 1 year) would be necessary to assess the efficacy of the immunomodulatory therapy.

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Table 1: Clinical Classification and Investigator Assessment Form (IAF) Mean Symptom Severity Score.

Patient CRF No.	Clinical Classification (Stratification)	Patient Self-report to Investigator ¹ (Where Objective =O and Subjective = S)	IAF Mean Symptom Severity Score ²		
			Week 0	Week 12	%Change Week 12 v. 0
16	Improved	O: Sleep quality improved. Decreased shortness of breath, decreased palpitations S: Muscle pain, memory improved.	1.63	0.90	-45%
14	Improved	O: Lymph nodes were less tender and smaller. S: Irritability, difficulty of thinking and concentrating decreased.	1.80	1.00	-44%
3	Improved	O: Returned to work part-time. S: Memory, generalised fatigue, muscle weakness and discomfort improved.	1.75	1.15	-34%
9	Improved	O: Returned to work full-time (had not been to work for 2 years). Chronic eczema ceased. Speech, balance improved. S: Pre-trial severe headaches, level of pain, sweats, social functioning, fatigue, memory improved.	1.90	1.30	-32%
8	Improved	O: Able to continue to work. S: Memory, anxiety, general strength improved.	1.84	1.35	-27%
4	Improved	O: Sleep improved. S: Memory improved, felt more energised, tolerance of emotional difficulties increased.	1.55	1.45	-6%
12	Not-Improved	No Improvement	1.25	1.10	-12%
7	Not-Improved	No Improvement	0.89	0.80	-11%
1	Not-Improved	No Improvement	1.35	1.25	-7%
10	Not-Improved	No Improvement	0.85	0.80	-6%
Group Results					
Median (SD) Improved % Change Week 12 v. Week 0					-32.9% (14.16)
Median (SD) Not-Improved % Change Week 12 v. Week 0					-8.8% (2.73)

Notes:

¹ **Patients self-report to investigator:** At the end of the blind phase, patients reported to the investigator about their work capacity, daily activity, social function, subjective sense of control over symptoms, fatigue, duration of complaints.

² **IAF Mean Symptom Severity Score:** Study specific 20-item list of CFS – related symptoms.

Table 2. Median cognitive deficit subset scores of the Symptom Checklist Questionnaire (SCL-90-R) during the blind phase (week 0-12).

Blind phase week 0-12	Median (SD)			Kruskal-Wallis
	Improved	Not-Improved	Placebo	p
Patient N	6	4	5	
Cognitive score Week 0	1.875 (0.85)	1.313 (0.43)	2.50 (0.63)	0.05
Cognitive score Week 12	1.875 (0.62)	1.375 (0.82)	2.25 (0.63)	0.25
Change Week 12 over Baseline	-0.375 (0.42)	0.063 (0.41)	-0.375 (0.23)	0.43
% change Week12 over Baseline	-16% (38.1)	5% (32.5)	-11% (9.4)	0.73

(upper limit of normal = 1.5)

Table 3. Median Karnofsky performance scores at baseline (week 0) with change of status indicated at week 12

Blind phase week 0-12	Improved	Not-Improved	Placebo	Kruskal-Wallis
				p
Patient N	6	4	5	--
Baseline KPS Score Median (SD)	62.5 (4.08)	65.0 (4.08)	60.0 (4.18)	0.46
% Change Week 12 v. 0 Median (SD)	0.6% (12.1)	0.0 (10.7)	3.0% (6.9)	0.93
No. of patients decreased (%change)	0	1 (-14%)	0	--
No. of patients increased (%change)	2 (+17%, +17%)	1 (+17%)	0	--

A change of the status occurs if the score change ≥ 10 points

Table 4. Median scores on the fibromyalgia tender point test, Week 12 Over Baseline.

Blind phase week 0-12	Median (SD) Score			Kruskal-Wallis
	Improved	Not-Improved	Placebo	p
Patient N	6	4	5	--
Fibromyalgia tender points Week 0	12.5 (6.6)	10 (8.5)	16 (1.4)	0.44
Fibromyalgia tender points w12	10 (6.6)	8.5 (10.1)	17 (4.1)	0.40
Change Week 0 over Baseline	-0.5 (3.1)	0 (2.5)	0 (3.56)	0.74
% change Week 12 over Baseline	-3.3% (25.4)	0.5% (52.5)	0% (22.4)	0.84

Table 5. Differences in NK cell numbers and NK cell activity between groups at Baseline, 12 and 16 weeks.

Variable	Median (SD) Improved	Median (SD) Not-Improved	Median (SD) Placebo	Kruskal-Wallis p
Week 0				
NK Cell #	134.0 (97.4)	204.0 (89.1)	162.0 (62.6)	0.78
NK activity LU-5%	6.0 (3.7)	7.8 (4.7)	6.3 (0.8)	0.96
NK activity LU-15%	1.65 (0.8)	3 (1.2)	1.3 (0.5)	0.44
Week 12				
NK Cell #	229.5 (133.7)	244.0 (119.8)	97.0 (89.5)	0.18
NK activity LU-5%	23.7 (7.5)	14.7 (3.4)	10.6 (4.0)	*0.03
NK activity LU-15%	6.4 (2.2)	3.7 (0.5)	3.0 (0.7)	*0.03
Week 16:				
NK cell #	269.5 (139.3)	224.5 (98.1)	127.0 (95.2)	0.23
NK activity LU-5%	12.8 (8.7)	10.1 (9.4)	7.5 (4.1)	0.52
NK activity LU-15%	3.7 (2.4)	2.3 (2.6)	1.95 (1.2)	0.68

*significant at $p \leq 0.05$ level**Table 6. NK cell activity changes within each treatment group (Wilcoxon matched pairs signed rank test p-value)**

Variable	Week 12 – Week 0	Week 16 – Week 0	Week 16 – Week 12
NK Cell #			
Improved	0.09	0.09	0.46
Not Improved	0.11	0.59	0.47
Placebo	0.35	0.50	0.22
NK activity LU-5%			
Improved	0.07	0.07	*0.04
Not Improved	0.18	0.59	0.19
Placebo	0.32	0.29	0.11
NK activity LU-15%			
Improved	0.07	0.07	*0.04
Not Improved	0.32	0.65	0.18
Placebo	0.32	0.29	0.65

*significant at $p \leq 0.05$ level

Table 7. Analysis of T cell subsets and T helper cytokines (IL-1a, IL-2, IL-10, IL-12 and IFN-g) by PBMC from CFS patients following stimulation with PHA or anti-CD3 antibodies: Tests of differences between groups at week 12.

Variable	Median (IQR) Improved		Median (IQR) Not-Improved		Median (IQR) Placebo		Kruskal- Wallis p
CD4	995.5	(888, 1114)	659.5	(510, 816)	575	(475, 780)	* 0.03
CD8	324.0	(273, 458)	283.5	(245, 427)	259.0	(250, 398)	0.62
HLA-DR	401.5	(336, 472)	263.5	(244, 323)	227.0	(213, 310)	0.19
IL1a PHA	0.0	(0, 0)	0.0	(0, 0)	0.0	(0, 0)	--
IL1a anti-cd3	0.0	(0, 0)	0.0	(0, 12)	0.0	(0, 95)	0.65
IL2 PHA	1457.5	(989, 1898)	1150	(729, 1790)	712	(603, 985)	0.21
IL2 anti-cd3	21.5	(0, 67)	74.5	(0, 161)	0.0	(0, 0)	0.68
IL10 PHA	298.5	(258, 534)	272.5	(175, 480)	260.0	(125, 299)	0.48
IL10 anti-cd3	205.5	(143, 311)	204.5	(162, 272)	125.0	(109, 135)	0.08
IL12 PHA	0.0	(0, 0)	0.0	(0, 0)	0.0	(0, 0)	0.87
IL12 anti-cd3	0.0	(0, 0)	0.0	(0, 34)	0.0	(0, 0)	0.96
IFN γ PHA	450.0	(364, 908)	266.5	(157, 2257)	827.0	(233, 1676)	0.86
IFN γ anti-cd3	247.0	(170, 388)	298.5	(140, 859)	492.0	(175, 505)	0.88

* significant at $p \leq 0.05$ level

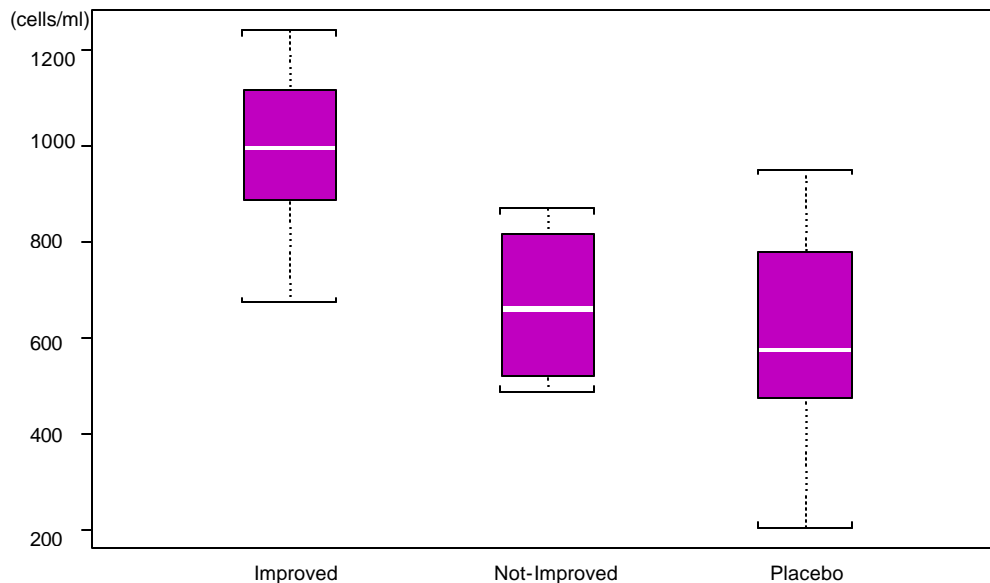


Figure 1. Box plots of CD4+ T helper cell numbers by group at week 12. Treatment with Isoprinosine® resulted in significantly greater numbers of CD4+ T helper cells in the Improved Group at week 12. Boxes indicate interquartile range, the middle line indicates the median, and the whiskers indicate maximum and minimum values. Kruskal-Wallis test for difference between groups at week 12 $p < 0.03$ (medians are Improved 995.5, Not-Improved 659.5, Placebo 575).

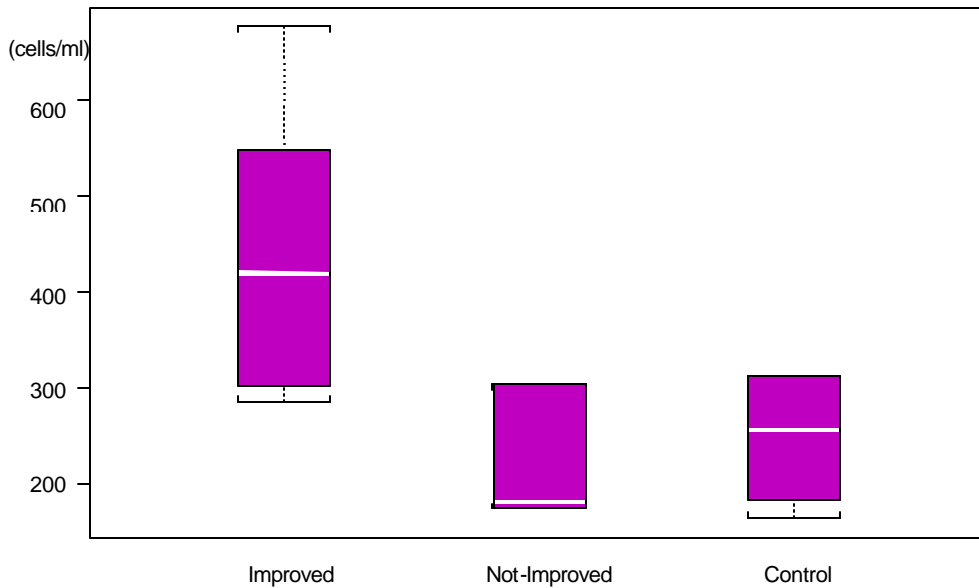


Figure 2. Box plots of CD4+ HLA-DR+ T Cell Numbers by group at week 28. Treatment with Isoprinosine® for 28 weeks resulted in an increased number of CD4+ HLA-DR+ T cells. Boxes indicate interquartile range, the middle line indicates the median, and the whiskers indicate maximum and minimum values. Kruskal-Wallis test for difference between groups at week 28 $p < 0.05$ (Medians [IQRs] are Improved 420 [303, 550], Not-Improved 182 [174, 304], Control 257 [183.5, 312.5]).

Appendix A.

Symptom Checklist-90-R (SCL-90-R) Cognitive subset questions

How much were you distressed by:		Not at all	A little bit	Moderately	Quite a bit	Extremely
1	Headaches					
2	Trouble remembering things					
3	Temper outbursts that you could not control					
4	Having to do things very slowly to insure correctness					
5	Having to check and double-check what you do					
6	Your mind going blank					
7	Trouble concentrating					
8	The idea that something is wrong with your mind					

Appendix B

Investigator Assessment Form

PATIENT NUMBER:						PATIENT INITIALS:					
CIRCLE VISIT ← (BELOW)											
Baseline		End of Week 4		End of Week 8		End of Week 12		End of Week 16		End of Week 28	
SIGNS AND SYMPTOMS						SEVERITY (if present)					
						Absent		Mild		Moderate	
1	Sore throat										
2	Painful cervical or axillary lymph nodes										
3	Unexplained, generalized muscle weakness										
4	Muscle discomfort or aches										
5	Prolonged (24 Hour+) generalized fatigue										
6	Generalized headaches										
7	Migratory painful joints without swelling or redness										
8	Non-exudate inflammation of pharynx										
9	Palpable or tender cervical or axillary lymph nodes										
10	Areas of lost or depressed vision										
11	Visual intolerance of light										
12	Forgetfulness										
13	Excessive irritability										
14	Confusion										
15	Difficulty thinking										
16	Inability to concentrate										
17	Depression										
18	Excessive sleep										
19	Inability to sleep										
20	Mild fever (oral: 99.5-101°F)										