Clin Drug Investig (2014) 34:553–564
DOI 10.1007/s40261-014-0208-z

ORIGINAL RESEARCH ARTICLE

Long-Term Inhaled Granulocyte Macrophage–Colony-Stimulating Factor in Autoimmune Pulmonary Alveolar Proteinosis: Effectiveness, Safety, and Lowest Effective Dose

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Published online: 3 June 2014

Abstract

Background and Objectives Granulocyte macrophage–colony-stimulating factor (GM-CSF) causes variable improvement in autoimmune pulmonary alveolar proteinosis (aPAP). Upon response to short-term treatment, patients are divided into responders and non-responders. The aim of this study was to test the hypothesis that long-term inhaled GM-CSF (iGM-CSF) is effective in all patients and that attainment of remission permits gradual de-escalation of the dose to the lowest effective safe dose.

Methods Patients were treated with iGM-CSF 250 μg once a day given 4 days on and 4 days off for as long as necessary (the "as far as it takes" protocol). Upon remission, defined as absence of symptoms, oxygen desaturation <4 % at the walking test, and significant radiographic reduction of the infiltrates, or at least two of the above, the iGM-CSF dose was de-escalated. In the case of relapse, the patient was repositioned at the previous effective dose. Patients were investigated at 6-month intervals. To detect hematopoietic effects, blood cell counts, CD34+ cells, granulocyte macrophage progenitor colony-forming-units, and burst-forming-unit erythroid were measured.

Results Six (five female) patients 43.8 ± 15.7 years of age were treated for 14–65 months and all responded to

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treatment. Remission was achieved after 25.6 ± 10 months. Three patients maintained remission at their lowest effective dose. Two patients relapsed at de-escalating doses. One patient remains on full-dose treatment. iGM-CSF had no impact on any of the hematological parameters tested.

Conclusions In aPAP, long-term adherence to the dose schedule permitted remission in all patients. Long-term treatment with iGM-CSF also permitted the definition of lower effective doses, minimizing disease burden and treatment costs safely, since no stimulating activity on hematopoiesis was observed, a fact that is of paramount importance for those aPAP patients needing lifelong treatment.

Key Points

Inhaled administration of granulocyte macrophage–colony-stimulating factor (GM-CSF) proved effective in a variable proportion of patients with autoimmune pulmonary alveolar proteinosis (aPAP). Upon response to short-term treatment (24 weeks), patients are divided into responders and non-responders

The results of the present study showed that the application of a different long-term treatment schedule of inhaled GM-CSF for aPAP using 250 μg once a day in a 4 days on and 4 days off schedule for as long as necessary (the "as far as it takes" protocol) permitted disease remission

De-escalation of the doses after attaining remission was possible, minimizing disease burden and treatment costs safely since no stimulating activity on hematopoiesis was observed, a fact that is of paramount importance for those aPAP patients needing lifelong treatment

1 Introduction

Pulmonary alveolar proteinosis (PAP) is an ultra-rare disease characterized by the inappropriate accumulation of surfactant in alveolar macrophages and diffusely in the airspaces [1–3]. Due to airspace occupation, the respiratory reserve gradually declines, leading to progressive breathlessness on exertion and hypoxemia, which leads to severe respiratory insufficiency and death [2, 3]. In 1994, two independent groups of investigators reported the development of PAP in mice lacking the granulocyte macrophage–colony-stimulating factor (GM-CSF) gene [4, 5]. Subsequently, it became evident that in humans the primary defect in the disease relates mainly to impaired surfactant catabolism by the alveolar macrophages in the case of functional impediment of GM-CSF, defects in its receptor expression [6, 7], or mutations on surfactant protein B or C genes rendering surfactant unrecognizable from alveolar macrophages [8]. The most common form of the disease (90 % of patients), autoimmune PAP (aPAP), is related to autoantibodies against GM-CSF blocking its bioactivity in the lung macrophages and impairing their ability to catabolize surfactant and handle microorganisms [9–11].

Though somewhat unexpected [12, 13], subcutaneous administration initially and, more recently, administration through inhalation of recombinant GM-CSF [inhaled GM-CSF (iGM-CSF)] has proved effective in a variable proportion of patients with aPAP, with the inhaled route of administration showing superiority [14–20]. However, criteria for induction of remission were never defined clearly, and the effectiveness and response to the administered regimen was based on a variable level of improvement in a few clinical, physiological, and imaging parameters. Correspondingly, criteria to define treatment failure were loosely defined and marginal improvement or no improvement in the above-mentioned parameters for a variable time period were considered enough to define patients as non-responders and thus confine them to the whole lung lavage (WLL) treatment option. A factor in the above decision is certainly the very high cost of the available treatment. Furthermore, it is known that prior WLL may help to improve response to the iGM-CSF, abbreviating the time period to a perceptible response, a parameter not adequately evaluated in previous studies categorizing patients as responders and non-responders. However, it is not clear whether non-responders need higher doses, more prolonged treatment, both, or a different approach, as well as which factors influence response or relapse [21]. This is of primary importance since concerns regarding the safety of administering hematopoietic growth factors, including GM-CSF, have been raised by previous studies as their prolonged administration has been associated with the development of hematologic malignancies [22, 23]. Concerns about toxicity might also relate to the inhaled administration of GM-CSF because, if absorbed systemically, a deleterious stimulating effect on hematopoiesis may occur.

In this study we retrospectively examined whether long-term administration of iGM-CSF at a relative moderate dose of 250 μg once a day for 4 days on and 4 days off for as long as necessary (the "as far as it takes" protocol) is effective in attaining remission in all patients with aPAP, and whether the attainment of remission by strict criteria such as the absence of symptoms, oxygen desaturation less than 4 % at the 6 min walk test (6MWT), and significant radiographic reduction of the infiltrates, or at least two of these factors, may permit adjustment of the inhaled dose to even lower levels, searching for "the lowest effective

dose." Furthermore, regarding safety concerns, we examined whether this intermediate dose schedule plus the eventual attainment of "the lowest effective dose" ensures safety by performing tests of hematopoietic toxicity, such as measuring complete blood cells counts, CD34+ cell counts, granulocyte macrophage progenitor colony-forming-units (GM-CFU), and burst forming unit erythroid (BFU-E) before and after iGM-CSF administration.

2 Patients and Methods

2.1 Study Design

This is a retrospective analysis of all patients with aPAP referred to the 2nd Pulmonary Department, "Attikon" University Hospital, Athens, Greece. Four patients were lost to follow-up before starting inhaled treatment and one patient presented spontaneous remission. The six remaining patients were treated with iGM-CSF. It is of note that all patients treated with iGM-CSF were done so not in the context of a prospective clinical trial but for rescue therapy. More precisely, most patients had already been treated with WLL, which is still considered the standard of care for patients with PAP of any etiology [24]. WLL was performed in a consistent and reliable manner, mostly in referral centers in Europe; this aided survival and delayed progression of disease but also raised considerable social and financial considerations due to the need for the patient to travel abroad and have at least one accompanying family member with him/her during consultations and treatment. All patients were symptomatic and presented with either no response or relapse of the disease after repeated WLL sessions. Two patients had been successfully treated with subcutaneous administration of GM-CSF a few years ago (one was lost to follow-up). As a result, one of these two patients was the first to ask for the option of iGM-CSF treatment. Following the initial patient, patients unsuccessfully treated with WLL were informed about the option of being treated with iGM-CSF based on existing evidence and were referred to our clinic from all over the country. Since iGM-CSF is not officially approved by European Medicines Agency for the treatment of aPAP, patients were informed that the medication would be imported and its use for aPAP approved by the Greek National Drug Administration and Ministry of Health as well as the Scientific Committee of the Hospital on the basis of the existing legislation for the use of "off-label" drugs and the scientific evidence already published in the literature [19, 20]. To initiate this off-label rescue therapy, all patients were consecutively registered at the Greek National Drug Administration with a specific registration number (e.g., 10088/22-1-2008, 79458/24-11-2010, 82847/8-12-2010, 200054/2011, 29840/26-4-2012), written informed consent was obtained from each patient, and the study was approved by the Medical Ethics Committee of "Attikon" University Hospital, Athens, Greece.

2.2 Subjects

Eleven patients (three males and eight females) were evaluated. Six of the 11 patients were treated with iGM-CSF when the drug became available. Diagnosis was documented by cytology examination of bronchoalveolar lavage or by histopathology examination of lung biopsy. The autoimmune nature of PAP was proven by the presence of neutralizing anti-GM-CSF antibodies in serum [25]. After treatment initiation, follow-up data taken at 6-month timepoints were collected and compared with the initial evaluation for each patient.

2.3 Treatment

All patients received treatment with iGM-CSF (Leukine® Sargramostim; licensed to Genzyme Corporation, Sanofi-Aventis U.S. LLC, Cambridge, MA, USA). GM-CSF was dissolved in sterile saline and inhaled as an aqueous aerosol using an I-neb Adaptive Aerosol Delivery (AAD) system (by Philips-Respironics). All patients were initially treated with iGM-CSF at a dose of 250 μg once a day for 4 consecutive days (4 days "on") and then 4 days "off" independent of their sex or bodyweight. The dose of 250 μg was chosen based on the international experience already published [20, 21], whereas the schedule of "4 days on/4 days off" was based first on our previous experience with subcutaneous administration of GM-CSF, second on the experience of the European center that referred the first patient to us for treatment with iGM-CSF and had already successfully applied the "4 days on/4 days off" schedule, and, finally, on the notion that administration of hematopoietic growth factors causes peak mobilization of hemopoiesis on days 4, 5, and 6 after initiation of daily treatment [26]. By choosing the 4 days on/4 days off schedule we hoped to minimize mobilization of hemopoiesis, if any might occur, after iGM-CSF treatment.

Upon remission, defined as (a) absence of symptoms, (b) oxygen desaturation less than 4 % at the 6MWT, and (c) significant radiographic reduction of the infiltrates, or at least two of the above, the administered dose was gradually decreased until relapse (reappearance of breathlessness on exertion, chest radiographic deterioration, and/or higher than 4 % desaturation on the 6MWT) and then repositioned to the previous de-escalating but effective dose schedule. Radiographic criteria appear to be loosely defined but the excessive cost of the treatment obliged the treating physician (SAP), upon fulfillment of the clinical and physiologic criteria and after significant

reduction but not complete resolution of the infiltrates, to move patients on to the lower dose step, usually one more day of "off" treatment. Patients were systematically submitted to chest radiography instead of chest computed tomography (CT) due the fact that the treatment period was much longer than the 12- to 24-week study period of already published studies and that some of our patients might require lifelong administration of iGM-CSF. As a result, we were very skeptical about performing a chest CT every 6 months or even sooner due to the increased risk of accumulated radiation dose side effects. If improvement persisted for several months, the next step was adding days without "on or off treatment" on a case-by-case basis, also considering the availability of the drug to the hospital pharmacy and the obtainment of approval from the health national service. Treatment was never suspended in any patient.

2.4 Monitoring During Treatment

All patients were monitored for compliance to treatment through reporting of vials of used medication. Peripheral blood counts and physical examination were performed at more or less regular intervals. Radiologic examination, pulmonary function [27], and exercise tests [28] were performed at 6-month intervals unless otherwise indicated by a significant change in the clinical status of the patient. Dyspnea was assessed using the Modified Borg Scale [29]. The disease severity score (DSS) was calculated for each patient at every timepoint of evaluation in the following manner, as already described in the literature [20]: DSS 1—no symptoms and partial pressure of oxygen in arterial blood (PaO_2) $\geq$70 mmHg; DSS 2—symptomatic and PaO_2 $\geq$70 mmHg; DSS 3—PaO_2 $\geq$60 and <70 mmHg; DSS 4—PaO_2 $\geq$50 and <60 mmHg; and DSS 5—PaO_2 <50 mmHg.

Systemic and hematopoietic toxicity was assessed both clinically and through systematic blood examination. In order to determine whether iGM-CSF is absorbed into the systemic circulation and if it has any systemic impact by stimulating hematopoiesis, we performed the following tests before and after 4 days of iGM-CSF administration: (1) complete blood count measurement using an automatic coulter and peripheral blood smears examined under light microscope; (2) the absolute number of CD34+ cells, to detect immature hematopoietic cells, was estimated by flow cytometry and with the use of standard protocols [3, 30]; measurement of GM-CFU, to detect progenitors committed to granulocytic-monocytic lineage; and BFU-E, to detect progenitors committed to erythroid lineage [30]. All of the above hematologic measurements were performed once in every patient at least 1 year after the initiation of iGM-CSF treatment.

2.5 Statistical Analyses

Variables are presented as mean ± standard deviation or median (interquartile ranges). Comparisons of variables between timepoints were performed using the Wilcoxon signed-rank test (for the comparison of data in two different timepoints) or Friedmans' test for repeated measures (for the comparison of data in three or more timepoints). Post hoc procedures were employed to identify significant differences over time. P values <0.05 were considered statistically significant. Data were analyzed using SPSS® version 17.0 for Windows (SPSS Inc., Chicago, IL, USA) and graphs were created with STATA® version 10 (StataCorp LP, College Station, TX, USA).

3 Results

3.1 Patient Characteristics

Six patients 43.8 ± 15.7 years of age were treated for 35.3 ± 18.1 months. Demographic, clinical, and functional characteristics of the patients are shown in Table 1. No specific occupational exposures were detected and the disease presented in almost all patients as progressive moderate to severe dyspnea on exertion and cough. One patient presented cachexia and desaturation upon minimal exercise (patient No. 1) and two other patients presented with severe respiratory failure at rest (patients no. 3 and no. 4), necessitating hospitalization.

3.2 Response to Treatment

Upon closing the study, all patients are still alive and in good clinical status. All patients but one had already undergone WLL sessions at a mean time of 4.4 months before initiation of iGM-CSF (Table 1). The treatment period ranged from 14 to 65 months (Table 1). All patients improved at variable timepoints from the beginning of treatment, as shown by improvements of DSS score, diffusing capacity of the lungs for carbon monoxide (DLCO), dyspnea according to the Borg scale, and alveolar–arterial oxygen difference ($P(A\text{-}a)O_2$) difference (Tables 2, 3; Figs. 1, 2, 3). They fulfilled remission criteria at a period of 25.6 ± 10 months (Table 4), as documented by the disappearance of dyspnea (Fig. 2), improvement of gas exchange at rest (Fig. 3), of arterial oxygen saturation (SaO_2) after 6MWT and of the radiographic infiltrates (Fig. 4a–f). All three patients presenting with respiratory failure at initiation of treatment recovered within 1 to 12 months. One patient (No. 1) had received all of the available therapeutic options during her long disease history (Figs. 5, 6). The administered dose was gradually

Table 1 Demographic, clinical, and functional characteristics of the patients at the time of treatment initiation. Treatment period ranges from 56 to 260 weeks

Characteristic	Patient						
	1	2	3	4	5	6	All[a]
Age at diagnosis (years)	17	31	14	48	44	65	36.5 ± 19.5
Age at treatment initiation (years)	24	41	21	51	45	65	41.2 ± 16.6
Sex	Female	Female	Female	Female	Female	Male	
Smoking habit	Never smoker	Never smoker	Never smoker	Never smoker	Smoker	Ex-smoker	
BMI (kg/m^2)	16.5	21.1	19.7	42.6	23.3	28.7	25.3 ± 9.4
FEV_1 (% predicted)	35.5	72.4	53.8	ND	95.7	68.5	65.2 ± 22.4
FVC (% predicted)	42.3	73.8	50.9	ND	93.9	69.6	66.1 ± 20.3
FEV_1/FVC	73.3	84.3	92.0	ND	87.0	76.3	82.6 ± 7.7
Borg Dyspnea Scale	8	2	10	9	4	3	6.0 ± 3.4
TLC (% predicted)	45.0	76.8	51.8	ND	69.2	65.5	61.6 ± 13.0
DLCO (% predicted)	30.0	33.0	18.9	ND	53.7	52.7	37.6 ± 15.1
$P(A\text{-}a)O_2$ (mmHg)	40.98	28.98	76.98	66.98	33.98	48.98	88.7 ± 11.3
Anti-GM-CSF Ab (μg/mL)	8.9	32.9	10.1	28.9	101.2	15.2	22.1(9.8, 49.9)
Months since last WLL	4	5	7	3	NA	3	4.4 ± 1.7
Total number of WLLs	65	14	4	3	0	1	3.5 (0.8, 26.8)

anti-GMCSF Ab granulocyte macrophage–colony-stimulating factor antibodies, *BMI* body mass index, *DLCO* diffusion capacity for carbon monoxide, *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *NA* not applicable, *ND* no data, *$P(A\text{-}a)O_2$* alveolar–arterial oxygen difference, *TLC* total lung capacity, *WLL* whole lung lavage

[a] Values are presented as mean (±standard deviation) or as median (interquartile range)

Table 2 Disease severity scores at every timepoint for all patients

Time of treatment	Patient					
	1	2	3	4	5	6
Before treatment	3	2	5	5	2	3
6 months of treatment	2	2	3	4	1	2
12 months of treatment	1	1	1	3	1	2
Remission	1	1	1	2	1	
Final measurement	1	1	1	4	2	

Disease severity score: DSS 1—no symptoms and PaO_2 ≥70 mmHg; DSS 2—symptomatic and PaO_2 ≥70 mmHg; DSS 3—PaO_2 ≥60 and <70 mmHg; DSS 4—PaO_2 ≥50 and <60 mmHg; and DSS 5—PaO_2 <50 mmHg [20]

DSS disease severity score, *PaO_2* partial pressure of oxygen in arterial blood

lowered and patients were treated with decreased doses of iGM-CSF for a period of 14.6 ± 7.1 months (Table 5). Two patients (patients No. 4 and 5) relapsed upon lowering of the initial 4 days "on"/4 days "off" treating dose. Relapse of patient No. 4 occurred 10 months after the first dose reduction on the 3 days "on"/5 days "off" schedule, while relapse of patient No. 5 developed 5 months after the second dose reduction (10 months from remission) on the 2 days "on"/5 days "off" schedule. The remaining three patients are still stable and the last recruited patient still receives the full dose.

3.3 Adverse Effects and Effects on Hematopoiesis

No significant adverse effects were observed. No cough, wheezing, desaturation, or chest pain occurred during or after iGM-CSF inhalation. No patient developed fever or any infection necessitating admission to the hospital. No solid organ or hematologic cancer developed. Systemic effects such as arthralgia, myalgia, or bone pain were reported only once by patient No. 1 almost 3 years after the initiation of treatment and subsided rapidly after administration of simple analgesics. Physical, radiologic, and

Table 3 Pulmonary function measurements for all patients

Parameter	Before treatments	6 months of treatment	12 months of treatment	Day of dose decrease	Final measurement	*P* value[a]
FEV_1 (% predicted)	65.2 ± 22.4	69.8 ± 12.3	74.6 ± 12.2	83.7 ± 12.3	79.3 ± 12.8	0.085
FVC (% predicted)	66.1 ± 20.6	70.1 ± 12.9	75.1 ± 13.2	82.9 ± 9.2	80.1 ± 10.1	0.215
TLC (% predicted)	61.7 ± 13.0	67.7 ± 7.3	67.0 ± 5.9	76.4 ± 4.8	76.4 ± 4.8	0.199
DLCO (% predicted)	37.7 ± 15.1	44.6 ± 11.4	48.9 ± 7.7	61.2 ± 10.9	59.9 ± 10.5	0.005
$P(A\text{-}a)O_2$	49.5 ± 18.9	32.1 ± 8.5	32.5 ± 8.2	28.7 ± 4.1	32.9 ± 16.7	0.031
Borg Dyspnea Scale	6.0 (2.8, 9.3)	1.0 (0.8, 2.3)	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	0.0 (0.0, 1.0)	0.003

Values are presented as mean (± standard deviation) or as median (interquartile ranges)

DLCO diffusion capacity for carbon monoxide, *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *$P(A\text{-}a)O_2$* alveolar-arterial oxygen difference, *TLC* total lung capacity

[a] Comparisons have been performed with Friedmans' test

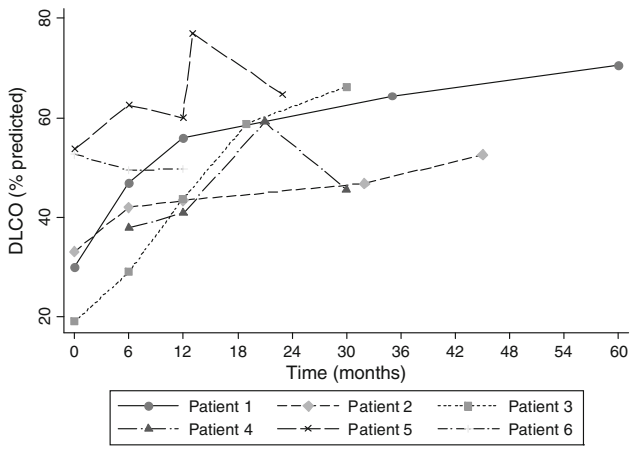

Fig. 1 Diffusion capacity for carbon monoxide of the six patients treated with inhaled granulocyte macrophage–colony-stimulating factor at five different timepoints: before treatment, after 6 months of treatment, after 12 months of treatment, at the time of disease remission, and on the last (current) measurement

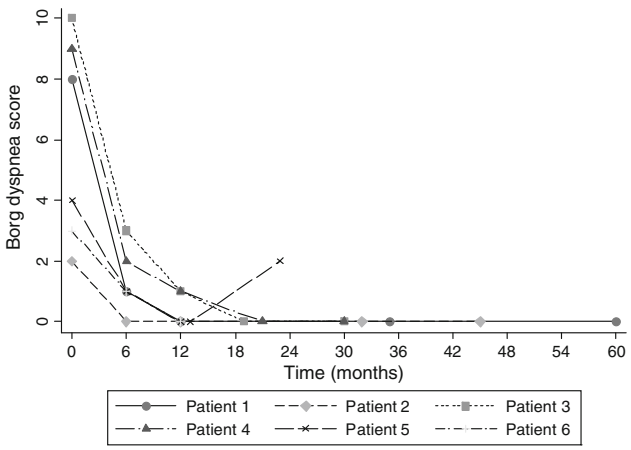

Fig. 2 Borg Dyspnea Scale score of the six patients treated with inhaled granulocyte macrophage–colony-stimulating factor at five different timepoints: before treatment, after 6 months of treatment, after 12 months of treatment, at the time of disease remission, and on the last (current) measurement

hematologic examination of this unique episode did not reveal any abnormalities.

All measurements for hematologic toxicity were performed at a median time of 18 months (range 9.75–39.5 months) after initiation of treatment. Administration of iGM-CSF for 4 consecutive days after at least 12 months on treatment had no impact on all hematological parameters tested (Table 6).

4 Discussion

The results of the present study show that long-term treatment with iGM-CSF 250 μg once a day given for 4 days on and 4 days off for as long as necessary (the "as far as it takes" protocol) permitted disease remission in all patients, avoiding non-responders. Furthermore, this study showed that achieving remission as defined by specific criteria permitted long-term stabilization or even further improvement after de-escalation of doses and allowed us to define lower effective doses in several patients, safely minimizing disease burden and treatment costs. To the best of our knowledge, this is probably the longest published experience with a high success rate of long-term iGM-CSF treatment in aPAP. In addition, this is also the first time that a search for lower effective doses of iGM-CSF has been attempted and attained, and this was proved safe as no stimulating activity on hematopoiesis was observed.

The results of this study confirm the overall effectiveness and safety of iGM-CSF for aPAP, as already shown [18–20]. Investigators had previously administered doses ranging from 125 to 500 μg/day for 24 weeks or, rarely, longer protocols of various durations of "on/off" days and showed that it is beneficial in 61.5–92 % of patients. However, in those treatment protocols patients were

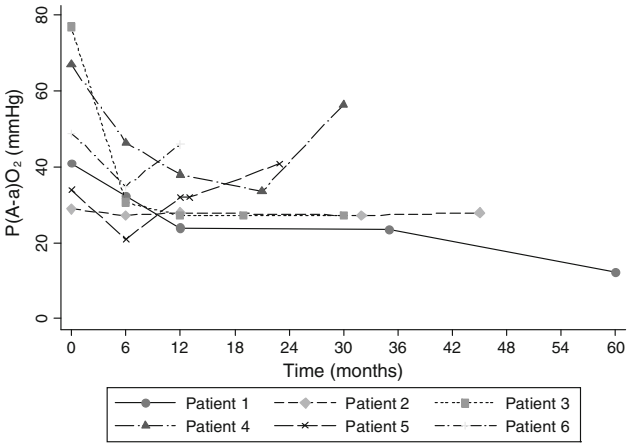

Fig. 3 Alveolar–arterial oxygen difference of the six patients treated with inhaled granulocyte macrophage–colony-stimulating factor at five different timepoints: before treatment, after 6 months of treatment, after 12 months of treatment, at the time of disease remission, and on the last (current) measurement

Table 4 Duration of treatment and time to remission in all patients treated with inhaled granulocyte macrophage–colony-stimulating factor

Patient	Total treatment time (months)	Time to remission (months)	Time from remission to the last measurements (months)
1	65	38	27
2	48	34	14
3	32	20	12
4	32	22	10[a]
5	24	14	10[a]
6	14	NA	NA
All (mean ± SD)	35.8 ± 18.1	25.6 ± 10.05	14.6 ± 7.1

NA not applicable, *SD* standard deviation

[a] Patients 4 and 5 have been considered to experience disease relapse

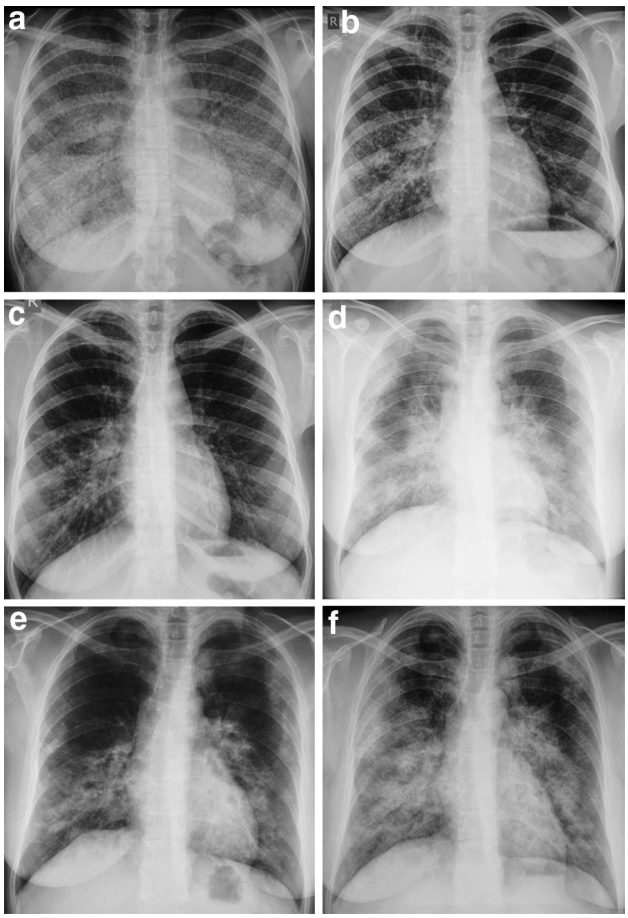

Fig. 4 Radiographic images of the chest of two of the six patients treated with inhaled granulocyte macrophage–colony-stimulating factor (iGM-CSF) at three different timepoints: before treatment, at the time of disease remission, and on the last (current) measurement. Images are considered representative of all patients. **a**–**c** Correspond to patient No. 3, who remains in remission and **d**–**f** correspond to patient No. 5, who developed disease relapse after the second diminution of the initial iGM-CSF dose

categorized as responders based on criteria such as the reduction of $P(A\text{-}a)O_2$ or the improvement in PaO_2 by at least 10 mmHg, and/or the increase of DLCO and forced vital capacity (FVC) at 12 and 7 %, respectively [19, 20]. Non-responders were allocated alternative treatment modalities. Previous studies on iGM-CSF also showed that response to treatment is dose and time dependent, discontinuation of treatment leads to relapse of the disease in a significant proportion of patients, reinstitution of iGM-CSF reacquires remission, and residual disease is almost always implied since radiographs and $P(A\text{-}a)O_2$ never normalize [19, 20].

This study adds to previous knowledge on treatment of aPAP by showing that the non-discontinuation of treatment and the application of the "as far as it takes" protocol of iGM-CSF permits patients to improve persistently in all clinical, functional, and imaging parameters, even though they presented with disease of greater severity than in previous studies [19, 20]. Half of our patients presented with severe respiratory insufficiency at initiation of treatment, in contrast to 0–40 % in other studies, and a far lower value of DLCO (37 % predicted, with one patient unable to perform due to severity) [19, 20]. Our decision to reduce the treating dose when improvement persisted for several months was fully justified by previous data showing that patients treated with "high-dose" protocols continue to improve at "low-dose" periods of treatment [20]. The search for lower effective doses was based on the systematic and close observation of the clinical, functional, and radiographic status of our patients at different timepoints as our protocol of adding days without "on or off treatment" on a case-by-case basis was applied. In addition, by using the "as far as it takes" strategy of treatment

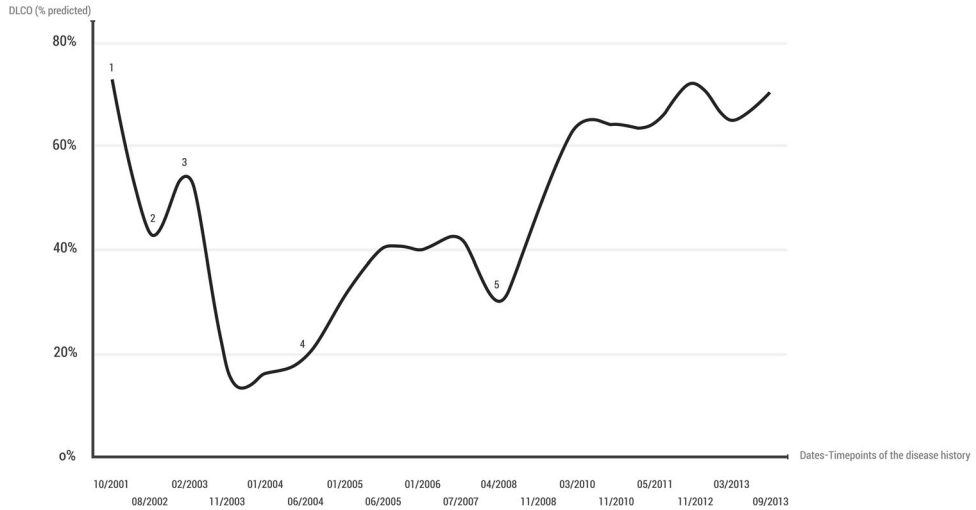

Fig. 5 Diffusing capacity of the lungs for carbon monoxide values for patient No. 1. (*1*) On 10/2001, the diagnosis of autoimmune pulmonary alveolar proteinosis (aPAP) was made. At this time the patient was asymptomatic and the pathological chest radiograph was an incidental finding during a pre-surgical examination for knee surgery. (*2*) During the next few months the patient deteriorated and became symptomatic and on 11/2002 subcutaneous granulocyte macrophage–colony-stimulating factor (GM-CSF) was administered intermittently. (*3*) In 9/2003, subcutaneous GM-CSF was withdrawn from the market and repeated whole lung lavage (WLL) sessions were initiated in London (65 WLL in total until the first administration of inhaled GM-CSF (iGM-CSF). (*4*) On 9/2004 lung infection by *Mycobacterium avium* was diagnosed and adequate treatment was administered plus the continuation of WLL. (*5*) On 5/2008, iGM-CSF was initiated and there has been gradual improvement to date: the patient is treated with a 3 days "on" and 7 days "off" dose schedule

three patients, those with the longest treatment experience (actually in lower-dose schedules), had almost normalized chest radiograms. This implies that full remission of disease in aPAP patients is attainable and may constitute the reference point for the definition of further treatment strategies.

Two patients relapsed at de-escalating doses: one on each of the 2 and 3 days "on"/5 days "off" treatment schedules. Detailed investigation in both cases showed that relapse in one case (patient No. 5) might be related to the reacquisition of a smoking habit and probably loss of compliance to treatment, and in the second case (patient No. 4) to the precipitous de-escalation of the dose schedule in relation to a delay in drug provision. In addition, the former patient was the only patient never treated by WLL, a factor that may have influenced her disease course. Relapse was not related a priori to the dose schedule since other patients still receive even lower doses. However, both relapsed patients never reached the magnitude of decline noted when they first started treatment, in contrast with patients in previous studies who had complete discontinuation of therapy and reached their baseline status [19, 20]. Although a treatment protocol such as ours has not been examined before, previous studies have reported relapse rates after the complete discontinuation of inhalation treatment ranging from 34.3 % at a mean time of 50.5 weeks [31] to 45 % at a mean time of 6.3 months [18]. Based on these data, it is difficult to explain which parameters determine response to treatment in aPAP since the present study reports relapses even under continuation of treatment, while the other two studies conclude that 55–65 % of patients remain free of disease for at least 30 months after complete discontinuation of treatment. Very few data exist concerning factors predicting response of aPAP to GM-CSF treatment. No differences in responses to iGM-CSF among patients of different disease severity groups and different GM-CSF neutralizing antibody levels have been reported [20]. Based on all of these observations and the lack of any significant and independent association of any of the epidemiologic, clinical, laboratory, and functional parameters with response to treatment and relapse [19, 20], one could state that the natural history of aPAP and its response to treatment follows a very unique route for each patient, necessitating close and watchful monitoring of any therapeutic interventions by the treating physician.

In the present study, all patients but one had already been treated with at least one session of WLL, attaining significant but not long-lasting benefit. WLL may benefit the subsequent responses to iGM-CSF [32], which might relate to the mechanical removal of engulfed macrophages, cellular debris, autoantibodies, cytokines, and surfactant leading at least to partial restoration of the alveolar microenvironment and facilitating iGM-CSF. The mechanism through which WLL benefits PAP patients has not been extensively studied, although two old studies report some favorable effect on alveolar

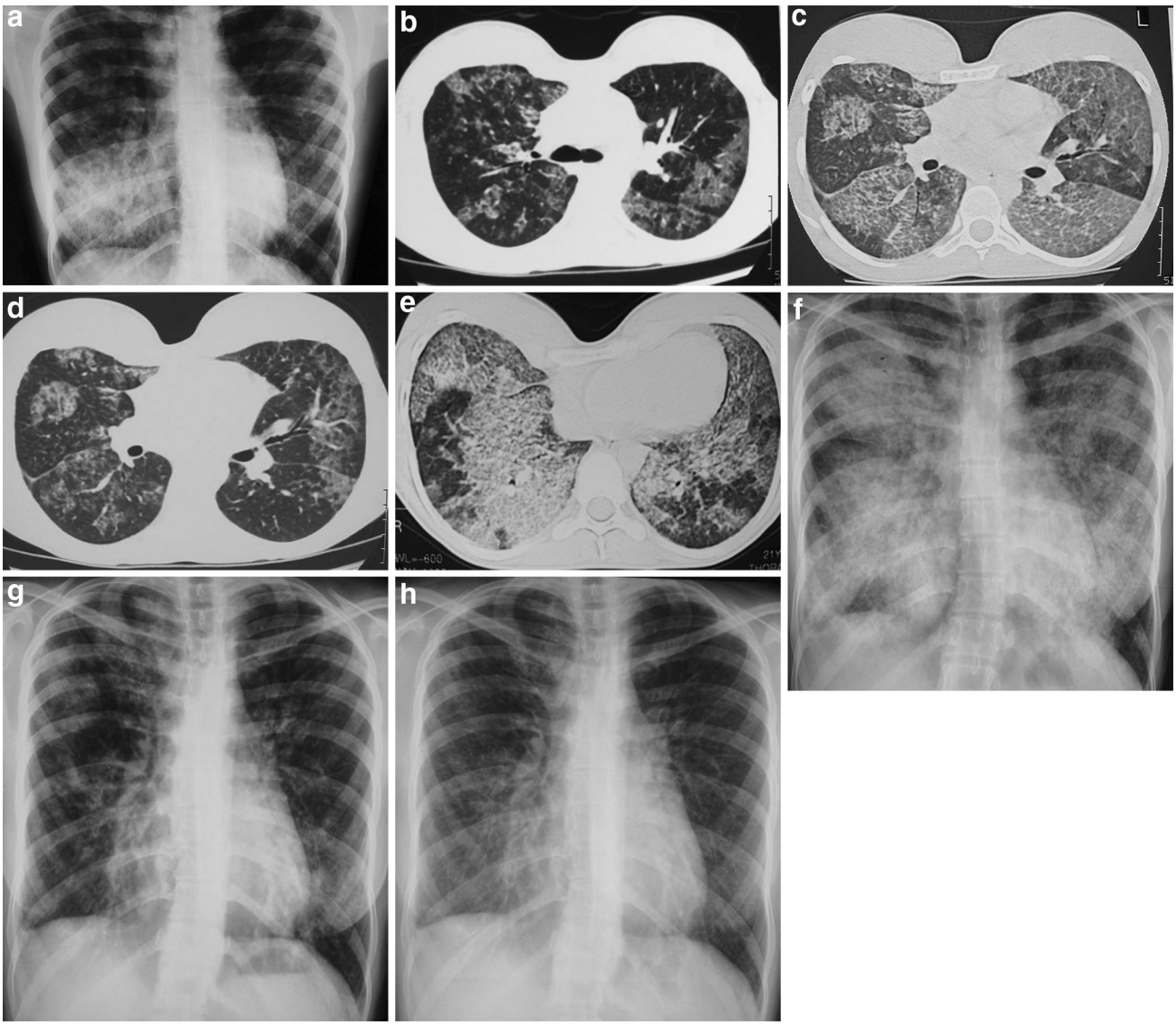

Fig. 6 **a**, **b** Radiographic and chest computed tomography (CT) images of patient No. 1 (Fig. 5; timepoints 1 and 2) at the time of diagnosis. Note the presence of pectus excavatum deformity and of patchy areas of ground glass attenuation and the sharp demarcation between normal and abnormal parenchyma. A ground glass opacity (GGO) score was calculated, as described by Tazawa et al. [20], and was found to be 3 (25–49 % GGO). **c** Significant deterioration of the infiltrates with extensive bilateral areas of ground glass attenuation and a superimposed fine linear pattern forming polygonal arcades with a GGO score of 5 (75 % or more GGO) (Fig. 5; timepoint 2). **d** Significant amelioration of the CT findings related to response to subcutaneous granulocyte macrophage–colony-stimulating factor (GM-CSF) treatment with a GGO score of 2 (Fig. 5; timepoint 3). **e** Severe clinical and radiologic deterioration despite repetitive whole lung lavage (WLL) sessions after withdrawal of GM-CSF from the international market, with a GGO score of 5 (Fig. 5; timepoint 4). **f**–**h** Radiographic images at initiation of inhaled GM-CSF treatment, at remission, and at the last evaluation, respectively. Significant progressive response to treatment becomes radiographically obvious with quasi-complete resolution of areas of consolidation (Fig. 5; timepoint 4)

macrophages function [33, 34]. On the other hand, iGM-CSF treatment has been shown to completely restore the alveoli microenvironment, the maturation markers, and the phagocytic ability of the macrophages [12, 35]. Despite important evidence regarding the efficacy and safety of iGM-CSF and the pathobiologic and not mechanical basis of this treatment, this option is considered as only an alternative to WLL and, most importantly, remains "off label," necessitating authorization of its use [21].

In this study, the intermittent treatment protocol with "days on and off" iGM-CSF was applied based on the observation that continuous administration could cause down-modulation of both granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) receptors on normal hematopoietic cells [36].

Table 5 Therapeutic dosage schedule of the six patients treated with inhaled granulocyte macrophage–colony-stimulating factor

Patient	Treatment initiation[a]		First dose decrease		Second dose decrease		Third dose decrease		Increase of dose because of relapse	
	Date	Dose	Date	Dose	Date	Dose	Date	Dose	Date	Dose
1	05/2008	4–4	07/2011	4–6	11/2012	4–7	03/2013	3–7		
2	10/2009	3–5	08/2012	2–6	07/2013	2–7				
3	02/2011	4–4	10/2012	2–6	04/2013	2–7				
4	02/2011	4–4	12/2012	3–5						
5	09/2011	4–4	10/2012	3–5	04/2013	2–5			09/2013	3–4
6	06/2012	4–4								

iGM-CSF inhaled granulocyte macrophage–colony-stimulating factor

[a] Treatment initiation consisted of 250 μg of iGM-CSF for 4 consecutive days followed by 4 days of no drug inhalation (i.e., 4 "on"/4 "off": 4–4) for all patients but one (No. 2) whose treatment was initiated with inhalation of iGM-CSF 250 μg for 3 days "on" and 5 days "off" (i.e., 3–5). Decreased doses are reported in the table using two numbers, the first of which refers to the number of consecutive days of "on" drug and the second to the number of days of "off" drug. In patient No. 5, because of disease relapse the dose was increased on 09/2013 (month/year) to 3–4 (i.e., 3 days of drug inhalation and 4 days without drug)

Table 6 Hematopoietic laboratory tests performed in all patients treated with inhaled granulocyte macrophage–colony-stimulating factor

Patients	Time in relation to treatment	WBC (/μL)	ANC (/μL)	LYM (/μL)	MON (/μL)	EOS (/μL)	CD34+ (/μL)	GM-CFU (/mL)	BFU-E (/mL)
1	Before	7,150	3,680	2,500	350	270	1	25	15
	After	6,190	3,490	1,840	330	250	1	25	12
2	Before	4,450	2,200	1,740	210	120	1	26	10
	After	6,250	3,490	2,250	270	90	1	30	13
3	Before	4,470	2,920	1,050	300	50	2	20	16
	After	5,650	3,650	1,360	370	60	2	16	11
4	Before	7,640	4,220	2,370	440	320	2	12	30
	After	7,420	4,350	2,090	350	320	2	22	18
5	Before	7,070	3,630	2,280	270	620	2	28	14
	After	4,930	2,890	1,120	190	550	1	42	3
6	Before	6,580	2,720	3,380	290	70	2	36	4
	After	7,410	4,280	2,600	370	50	2	48	4

ANC absolute neutrophil count, *BFU-E* burst forming unit erythroid, *EOS* eosinophils, *GM-CFU* granulocyte-macrophage progenitor colony forming units, *LYM* lymphocytes, *MON* monocytes, *WBC* white blood count

Concerns regarding long-term administration of hematopoietic growth factors such as GM-CSF have been raised by previous studies in patients with malignant disorders as well as in congenital neutropenia [37, 38]. Transient cytogenetic abnormalities have been discovered in healthy donors who received G-CSF for stem cell mobilization [39]. We hypothesized that if a significant amount of iGM-CSF is absorbed in systemic circulation then stimulation of hematopoiesis has to occur and would be reflected by an increase of stem and progenitor cells, as already shown in previous studies when GM-CSF was systematically administered [40]. In this study, we did not observe any effect of iGM-CSF on any of these parameters, and therefore we assumed that the systemic impact of iGM-CSF should be minimal, if there was any at all. The absence of any stimulating activity on hematopoiesis of iGM-CSF is a very important observation if we consider that lifelong administration of GM-CSF might be required at least for some patients with aPAP.

The use of iGM-CSF to treat PAP remains "off-label." Evidence regarding it is based on a few observational studies and case reports since no randomized controlled trial has reported on the efficacy and safety of GM-CSF either as primary therapy or as an adjunct to WLL, probably due to the rarity of the disease. However, the high cost and the "off-label" use of such a treatment creates many ethical, legal, and policy problems, which are shouldered so far only by patients and doctors and not by health authorities. We believe that this small observational study adds new data on the efficacy and safety of iGM-CSF in aPAP that might further encourage prospective, randomized controlled trials to be conducted for the official approval of the drug as the mainstay of treatment in aPAP [41].

The limitations of the present study are mainly related to the small cohort of patients, which does not allow statistical analysis to better define the predictive factors for response to treatment and relapse of the disease. However, this single-country, singe academic center experience for an ultra-rare disease has permitted the pilot examination of a homogeneous population managed as uniformly as possible with a treatment protocol not ever applied before in search of data regarding its effectiveness, safety, and the lowest effective dose of iGM-CSF in aPAP.

5 Conclusion

The results of the present study show that long-term treatment with iGM-CSF permitted disease remission in all patients, avoiding non-responders. Furthermore, this study showed that the attainment of remission permitted long-term stabilization or even further improvement despite gradual de-escalation of the doses. This allowed us to define lower effective doses in several patients, safely minimizing disease burden and treatment costs, since no stimulating activity on hematopoiesis was observed, a fact that is of paramount importance for those aPAP patients needing lifelong treatment.

Acknowledgments This study is dedicated to the National Health System (ΕΣΥ) and to the people of Greece who, despite a quite catastrophic economic crisis, still support patients with severe and rare diseases to live. We thank Mrs. Andrea Schams for her help with determination of the GM-CSF autoantibodies. This work was supported in part by the German Federal Ministry of Education and Research (EuPAPNet project inside ERARE, Number 01GM1011A). We sincerely thank Mr. A. Analitis for statistical assistance.

Conflict of interest statement Matthias Griese was supported in part by the German Ministry of Education and Research (EuPAPNet project inside ERARE, Number 01GM1011A). Spyros A. Papiris, Panagiotis Tsirigotis, Likurgos Kolilekas, Georgia Papadaki, Andriana I. Papaioannou, Christina Triantafillidou, Anastasia Papaporfyriou, Anna Karakatsani, Konstantinos Kagouridis, and Effrosyni D. Manali have no conflict of interest related to this work.

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