

チェックする。使用薬剤と培養施設は GMP 準拠の国立循環器病センターの細胞処理センターで行われる。

G. 研究発表

1. 論文発表

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Human umbilical cord provides a significant source of unexpanded mesenchymal stromal cells.

Kikuchi-Taura A, Taguchi A, Kanda T, Inoue T, Kasahara Y, Hirose H, Sato I, Matsuyama T, Nakagomi T, Yamahara K, Stern D, Ogawa H, Soma T.

2. 学会発表

なし

H. 知的財産権の出願・登録状況 (予定を含む。)

1. 特許取得

卵膜、臍帯由来 MSC の採取法を特許につなげたいと考えているが、未出願である

2. 実用新案登録

何らかの臍帯細胞分取ツールを申請したい。

3. その他

厚生労働科学研究費補助金（難治性疾患等克服研究事業
（免疫アレルギー疾患等予防・治療研究事業）

分担研究報告書

卵膜由来間葉系幹細胞を用いた炎症性腸疾患の新規治療法の開発

研究分担者 大西 俊介 北海道大学大学院医学研究科 消化器内科 助教

研究要旨

ラット重症腸炎モデルに対して、ヒト卵膜由来間葉系幹細胞を投与したところ、著明に改善した。単球・マクロファージの活性化が抑制されていることが機序の一つとして考えられた。現在、臨床研究を開始するためのプロトコール作成を行っている。

A. 研究目的

炎症性腸疾患に対する新規治療法の開発へむけて、ヒト卵膜由来間葉系幹細胞(MSC)の効果およびその機序を明らかにする。

B. 研究方法

帝王切開による胎児娩出後の卵膜を母親の同意を得て採取し、MSCを分離・培養した。7週齢の雄性SDラットに対して8% dextran sulfate sodium (DSS)を5日間経口投与し、DSS投与開始2日目にMSC(1×10^6 cells)を静注した。細胞投与後4日間の体重の変化および臨床的重症度を評価した後、屠殺して大腸の長さ、病理スコア、直腸の炎症性サイトカインの発現を定量的RT-PCR法で測定した。

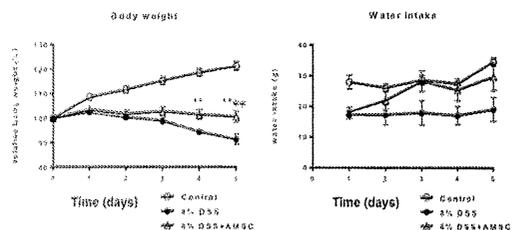
(倫理面への配慮)

ヒト卵膜の採取は天使病院の倫理委員会の承認を得て行った。また、ヒト卵膜からMSCを分離・培養し、動物モデルに投与することについては北海道大学医学部「医の倫理委員会臨床研究審査専門委員会」の承認を得て行った。

C. 研究結果

細胞投与群はコントロール群と比較して、体重減少、臨床的重症度、大腸の長さの短縮および病理スコアが有意に改善していた。直腸におけるTNF- α 、IL-1 β 、MIFなどの炎症性サイトカインの発現が有意に減少していた。さらに免疫組織染色では、単球/マクロファージのマーカーであるCD68陽性細胞の浸潤が有意に抑制されていた。

体重・飲水量の推移



厚生労働科学研究費補助金（難治性疾患等克服研究事業
（免疫アレルギー疾患等予防・治療研究事業））

分担研究報告書

「胎児付属物由来間葉系幹細胞の細胞製剤化を目指した研究」

研究分担者 名前 山原 研一 所属 国立循環器病研究センター

研究要旨：胎児付属物由来間葉系幹細胞（MSC）の臨床応用をめざし、その細胞製剤化を目指した検討を行った。結果、倫理委員会の承認の元、胎児付属物 MSC のセルプロセッシングセンター（CPC）での大量培養とその凍結保存法を確立し、品質管理を行い、更には PMDA 薬事戦略相談において、細胞製剤に必要な要件の確認を行った。

共同研究者

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JMCC. 2010;49:753-61, JMCC.
2012;53:420-8)。

これらを踏まえ、我々は胎児付属物 MSC を用い、各分担研究者と共同で、①同種造血幹細胞移植における GVHD、②Crohn 病、③膠原病、などの難治性免疫関連疾患を対象とした細胞治療の臨床応用を目指している。MSC を用いた細胞治療の特徴は、従来のステロイド、免疫抑制剤とは全く異なる機序により免疫抑制効果を発揮することであり、MSC は従来の治療法に抵抗性の難治性免疫関連疾患に対する新たな治療薬となる可能性が高い。そこで、本研究では、将来的な胎児付属物由来 MSC の製剤化を目指し、①胎児付属物 MSC のセルプロセッシングセンター（CPC）での分離、培養、凍結法の至適化、②CPC にて得られた胎児付属物 MSC を用いた安全性試験、③胎児付属物 MSC の製剤化を見据えた PMDA 薬事戦略相談、を行った。

A. 研究目的

近年 MSC はその免疫抑制能を有することで注目され、米国 Osiris 社は同種骨髄 MSC 「Prochymal」を用い、①造血幹細胞移植における重篤な副作用である移植片対宿主病(GVHD)、②Crohn 病など、従来の治療法ではコントロール不能な難治性免疫関連疾患を対象とした治験を行っている。

一方、我々は、胎児付属物 MSC の臨床応用を目指した研究をこれまでにっており、結果、胎児付属物 MSC は骨髄 MSC と比較し、①倫理的問題が少なく、②非侵襲的に採取可能であり、③非常に多くの細胞を一度に得られ、④免疫抑制効果も高い

B. 研究方法

①胎児付属物MSCのセルプロセッシングセンター（CPC）での分離、培養、凍結法の

至適化

国立循環器病研究センターCPC運営委員会および倫理委員会に対し、胎児付属物由来MSCの臨床試験用細胞調整に関する研究計画書を提出し、承認を受けた。同センター周産期婦人科の協力の下、母児共に健康な帝王切開例において、手術室にて無菌的に胎児付属物を採取し、同センターCPCに搬送した。CPC内で胎児付属物の羊膜(担当：山原) および臍帯(担当：兵庫医大相馬先生)を物理的に分離し、酵素処理にて組織消化した後、遠心操作にて沈殿する細胞成分を回収した。大型培養容器に播種し、数継代を経て細胞回収を行い、プログラムフリーザーにて凍結保存を行った。

②CPCにて得られた胎児付属物MSCを用いた安全性試験

臨床試験に使用する胎児付属物MSCの安全性試験として、薬食発第0912006号「ヒト(同種)由来細胞や組織を加工した医薬品又は医療機器の品質及び安全性の確保について」に基づき、下記の項目の検査を行った(先端医療センター橋本先生、東京医科歯科大学清水先生との共同研究)

- ・ 細胞数及び生存率
- ・ 確認試験
- ・ 細胞の純度試験
- ・ 無菌試験
- ・ マイコプラズマ否定試験
- ・ エンドトキシン試験
- ・ 下記に挙げるウイルス等の試験

B型肝炎(HBV)、C型肝炎(HCV)、ヒト免疫不全ウイルス(HIV)感染症、成人T細胞白血病、パルボウイルスB19感染症、サイトメガロウイルス感染、EBウイルス感染

・ 核型確認

③胎児付属物MSCの製剤化を見据えたPMDA薬事戦略相談

将来的な胎児付属物MSCを用いた治験導入を目指すには、胎児付属物MSCのGMPグレード製剤化が必要である。そこで、製剤化(医薬品化)に関し、PMDA薬事戦略相談を申請し、PMDAとの協議を行った。

(倫理面への配慮)

ヒト胎児付属物の採取、MSC樹立・培養・保存は、「臨床研究に関する倫理指針」を遵守し、当センターの倫理委員会の審査により承認後、提供者本人による書面での同意を得て実施した。また、提供者に対するインフォームド・コンセントは書面で行った。

C. 研究結果

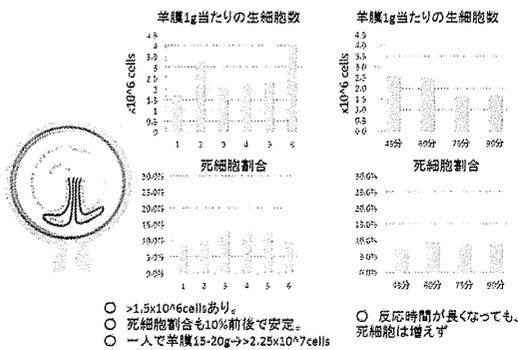
①胎児付属物MSCのセルプロセッシングセンター(CPC)での分離、培養、凍結法の至適化

(a)卵膜MSC分離工程

帝王切開にて得られた胎児付属物から、手動的に卵膜を分離し、更に羊膜および絨毛膜が再現性をもってそれぞれ15g以上および30g以上採取することが可能であることが分かった。

羊膜および絨毛膜それぞれから最も効率のおよび低コストにMSCを分離する至適条件を、使用酵素の種類、その濃度、および反応時間を調整し、検討を行った。結果、酵素組成を2種類とし、これら濃度および反応時間を至適化することで、目的とするMSCが卵膜1gあたり常に 10^6 個以上得られることを確認した(図1)：特許準備中。

ヒト卵膜の酵素処理による細胞収率:最近6回の検討



(図1) 酵素処理により得られるヒト羊膜由来細胞数 (1g 当たり)。

(b)胎児付属物 MSC 培養・凍結保存工程

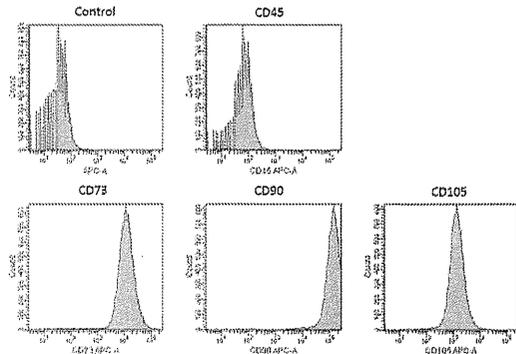
例えば急性 GVHD 治療に用いられる MSC は体重 kg 当たり 2×10^6 細胞であり、一回の静脈内投与に 2×10^8 細胞、計 4 回の投与とすると計算上 8×10^8 細胞と、 10^9 細胞オーダーの MSC を用意する必要がある。CPC にて実際に培養を行ったところ、市販の多段培養容器(Corning 社製セルスタック 10 チャンバー)一つで 10^8 細胞オーダーの卵膜 MSC を得られることが分かった。そこで同容器を用いて大量培養を行い、数人分の治療に用いることが可能な $>10^{10}$ 細胞数を凍結保存することが出来た。

②CPC にて得られた胎児付属物 MSC を用いた安全性試験

CPC 内で凍結保存された臨床試験用卵膜 MSC に関する安全性試験として、

- ・ 細胞数：規格細胞数の 90%以上
- ・ 生存率：全有核細胞の 90%以上
- ・ 確認試験：形態的に紡錘形
- ・ 細胞の純度試験：FACS による表面マーカー解析にて、CD73/90/105 陽性細胞の割合：99%以上、CD45 陽性細胞 0.1%未満 (図 2)

卵膜MSCの品質管理:表面マーカー解析



(図2) CPC 内大量培養羊膜 MSC の表面マーカー解析

であることを国立循環器病研究センター内で確認した。更に、

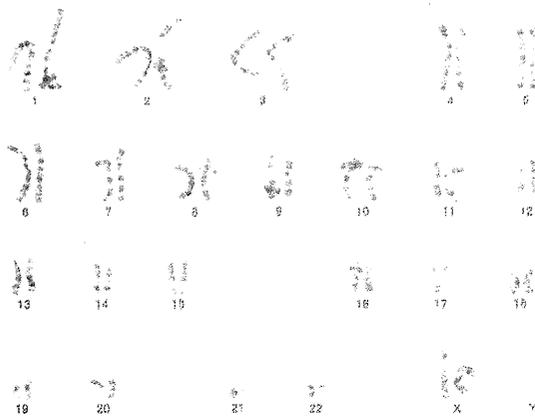
- ・ 無菌試験 (メンブレンフィルター法：陰性)
- ・ マイコプラズマ否定試験 (PCR 法・DNA 染色法：陰性)
- ・ エンドトキシン試験 (Kinetic-比色法：1.0EU/ml 以下)

に関しては、先端医療振興財団細胞療法開発事業部門細胞評価グループに外部委託し、常に試験合格であった。また、

- ・ ウイルス試験 (PCR 法：陰性)

を先端医療振興財団先端医療センター細胞治療科橋本先生および東京医科歯科大学難治疾患研究所清水先生のご協力の下行い、常に陰性であった。

- ・ 核型確認 (G-band 分析：正常 2 倍体)
- に関しては、日本遺伝子研究所に外部委託を行い、正常染色体であることを確認した (図 3)。



(図3) CPC 内大量培養羊膜 MSC の G-band 分析結果。

③胎児付属物 MSC の製剤化を見据えた PMDA 薬事戦略相談

胎児付属物 MSC の製剤化を目指し、それに必要な要件を確認するため、平成 24 年 10 月 25 日 PMDA 薬事戦略相談の対面助言を行った。結果、製剤化に関しては、細胞・組織製品の初回治験計画届書の調査 (30 日調査) を参考とすること、生物由来原料基準 (厚生労働省告示) を遵守すること、更に医薬品 GLP 省令を守ること、を指摘された。今後、細胞製剤化用の製品標準書、標準作業手順書を作成し、再度 PMDA の助言を受ける予定である。

D. 考察

本研究では、胎児付属物 MSC の細胞製剤化をめざし、CPC での簡便且つ大量培養法の開発、培養された MSC の安全性試験、更には製剤化に関する行政的な問題に関し、PMDA 薬事戦略相談の対面助言によって明確化を目指した。

我々の検討結果から、①胎児付属物 MSC の CPC 内での至適分離・大量培養法を樹立 (特許準備中)、②胎児付属物 MSC の安全

性試験の実施による品質確認、③胎児付属物 MSC の製剤化に必要な要件の整理、を終了できた。PMDA との協議から、胎児付属物 MSC の製剤化に必要な安全性・有効性評価項目が明らかとなり、今後精力的に製剤化に向けた製品標準書・標準作業手順書 (SOP) 等の必要書類の整備を行う予定である。

E. 結論

胎児付属物 MSC の細胞製剤化をめざし、その臨床試験グレードの大量培養と品質確認と、治験グレードに必要な要件確認を行った。今後、GMP グレードの細胞製剤化を目標とし、研究を進めていきたい。

G. 研究発表

1. 論文発表

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2. 学会発表（発表誌名巻号・頁・発行年等も記入）
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- H. 知的財産権の出願・登録状況（予定を含む。）
1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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IV. 研究成果の刊行物・別刷

Ghrelin Treatment of Cachectic Patients with Chronic Obstructive Pulmonary Disease: A Multicenter, Randomized, Double-Blind, Placebo-Controlled Trial

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Abstract

Background: Pulmonary cachexia is common in advanced chronic obstructive pulmonary disease (COPD), culminating in exercise intolerance and a poor prognosis. Ghrelin is a novel growth hormone (GH)-releasing peptide with GH-independent effects. The efficacy and safety of adding ghrelin to pulmonary rehabilitation (PR) in cachectic COPD patients were investigated.

Methodology/Principal Findings: In a multicenter, randomized, double-blind, placebo-controlled trial, 33 cachectic COPD patients were randomly assigned PR with intravenous ghrelin (2 µg/kg) or placebo twice daily for 3 weeks in hospital. The primary outcomes were changes in 6-min walk distance (6-MWD) and the St. George Respiratory Questionnaire (SGRQ) score. Secondary outcomes included changes in the Medical Research Council (MRC) scale, and respiratory muscle strength. At pre-treatment, serum GH levels were increased from baseline levels by a single dose of ghrelin (mean change, +46.5 ng/ml; between-group $p < 0.0001$), the effect of which continued during the 3-week treatment. In the ghrelin group, the mean change from pre-treatment in 6-MWD was improved at Week 3 (+40 m, within-group $p = 0.033$) and was maintained at Week 7 (+47 m, within-group $p = 0.017$), although the difference between ghrelin and placebo was not significant. At Week 7, the mean changes in SGRQ symptoms (between-group $p = 0.026$), in MRC (between-group $p = 0.030$), and in maximal expiratory pressure (MEP; between-group $p = 0.015$) were better in the ghrelin group than in the placebo group. Additionally, repeated-measures analysis of variance (ANOVA) indicated significant time course effects of ghrelin versus placebo in SGRQ symptoms ($p = 0.049$) and MEP ($p = 0.021$). Ghrelin treatment was well tolerated.

Conclusions/Significance: In cachectic COPD patients, with the safety profile, ghrelin administration provided improvements in symptoms and respiratory strength, despite the lack of a significant between-group difference in 6-MWD.

Trial Registration: UMIN Clinical Trial Registry C000000061

Citation: Miki K, Maekura R, Nagaya N, Nakazato M, Kimura H, et al. (2012) Ghrelin Treatment of Cachectic Patients with Chronic Obstructive Pulmonary Disease: A Multicenter, Randomized, Double-Blind, Placebo-Controlled Trial. *PLoS ONE* 7(5): e35708. doi:10.1371/journal.pone.0035708

Editor: Christian Taube, Leiden University Medical Center, The Netherlands

Received: December 9, 2011; **Accepted:** March 20, 2012; **Published:** May 1, 2012

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Funding: Funding was from the Research Grant from the Ministry of Health, Labour and Welfare in Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan; the Takeda Scientific Foundation in Japan; and Health and Labour Sciences Research Grants, Clinical Research, H20-translational research-general-002. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Pulmonary cachexia is common in the advanced stage of chronic obstructive pulmonary disease (COPD), and it is an independent risk factor for death in such patients [1,2]. Based on the notion that advanced COPD affects the whole body and causes wasting syndromes, many different therapeutic approaches have been attempted to improve this syndrome [1,3].

Pulmonary rehabilitation (PR) including exercise training is well accepted to improve exercise performance and quality of life in COPD patients [4], and it has been regarded as a nutritional adjunct therapy [5].

During the 1970s and 1980s, many gut peptides were identified [6]. Ghrelin, first discovered in 1999 as a novel growth hormone (GH)-releasing peptide isolated from the stomach, has been identified as an endogenous ligand for GH secretagogue receptor

[7]. Ghrelin also has a variety of GH-independent effects, such as causing a positive energy balance and weight gain by decreasing fat utilization [8], stimulating food intake [9], and inhibiting sympathetic nerve activity [10,11]. In addition, plasma ghrelin levels were elevated in cachectic COPD patients and were associated with the cachectic state and pulmonary function abnormalities, suggesting that endogenous ghrelin increased to compensate for the cachectic state and may provide important clues to improve the catabolic-anabolic imbalance in such patients [12]. In an open-label pilot study, we showed that ghrelin treatment increased walking distance in cachectic COPD patients [13]. Based on the above available evidence, a multicenter, randomized, double-blind, placebo-controlled study was conducted to test the hypothesis that the addition of ghrelin treatment to PR might benefit cachectic COPD patients. The objectives were to investigate the efficacy and safety of adding ghrelin to PR in cachectic COPD patients.

Methods

The protocol for this trial, supporting CONSORT checklist, and Supplementary Methods are available as supporting infor-

mation; see Protocol S1, Checklist S1, and Supplementary Methods S1.

Study Design and Patients

The study was a 3-week, multicenter, randomized, double-blind, placebo-controlled trial of ghrelin administration during PR. The study was finally conducted at four clinical centers (National Cerebral and Cardiovascular Center, Miyazaki University School of Medicine, Nara Medical University, and National Hospital Organization Toneyama National Hospital) in Japan from September 2005 through May 2009, because Graduate School of Medicine, Osaka City University did not participate just before the start of the clinical trial. The study was conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines and approved by the ethics committees of all participating study centers: The ethics committee of the National Cerebral and Cardiovascular Center (approval number, M17-13); The ethics committee of Miyazaki University School of Medicine (approval number, 218); The ethics committee of Nara Medical University (approval number, 05-012); and The ethics committee of the National Hospital Organization Toneyama National Hospital (approval number, 0311). All patients gave written

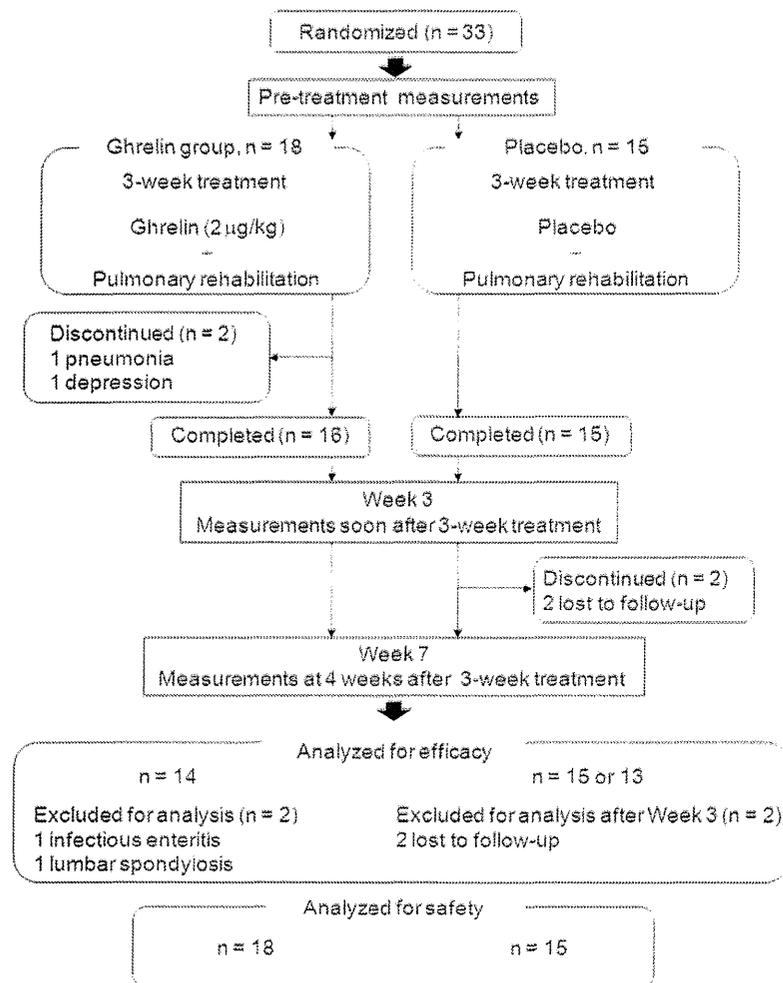


Figure 1. Trial profile.

doi:10.1371/journal.pone.0035708.g001

informed consent (in Japanese). The inclusion criteria were as follows: 1) severe to very severe COPD (forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) of less than 70% and FEV1 percent predicted of less than 50%); 2) underweight (body mass index (BMI) < 21 kg/m²); 3) clinically stable and able to participate in PR; 4) between 20 and 85 years old; and 5) signed the agreement for participation in this study. Participants were excluded for any of the following: 1) malignant tumors; 2) active infection; 3) severe heart disease; 4) hepatic dysfunction (serum aspartate aminotransferase and alanine aminotransferase levels at least twice the upper limit of normal); 5) renal dysfunction (serum creatinine levels \geq 2.0 mg/dl); 6) asthma; 7) definitely or possibly pregnant; 8) change in drug regimen within 4 weeks before participation in this study; or 9) judged to be unable to participate in this study by their physician. This study was registered with UMIN (University Hospital Medical Information Network in Japan: <http://www.umin.ac.jp/ctr/>), number C000000061.

Randomization and Interventions

Randomization was done in each center considered as a block. The randomization list was generated by a statistician from Hamamatsu University School of Medicine and maintained there

until the study was finished and unblinded. Neither the physicians nor the patients were aware of the treatment assignments. Patients who met the eligibility criteria were enrolled and randomly assigned in a 1:1 ratio to receive PR with either ghrelin (2 μ g/kg) or placebo twice a day for 3 weeks in hospital. The administration of ghrelin (2 μ g/kg, ghrelin solution with 10 ml saline) or placebo was done intravenously over 30 minutes at a constant rate and repeated twice a day for 3 weeks. Patients were tested at pre-treatment, Week 3 after start of ghrelin or placebo administration with PR, and Week 7 after start of ghrelin or placebo administration with PR, i.e., 4 weeks after the completion of the combination treatment (Figure 1).

Preparation of Human Ghrelin

Human ghrelin obtained from the Peptide Institute Inc. was dissolved in distilled water with 3.75% D-mannitol and sterilized as described previously [13]. Ghrelin was stored in 2-ml volumes, each containing 120 μ g ghrelin. The chemical nature and content of the human ghrelin in vials were rarefied as described previously [13]. All vials were stored frozen at -30°C until the time of preparation for administration.

Table 1. Patients' baseline characteristics. *

	Ghrelin, n = 14	Placebo, n = 15	p value
Age, years [†]	70.5 (6.2), 63–80	73.9 (6.0), 63–82	0.15
Sex, male/female [†]	13/1	13/2	1.00
BMI, kg/m ^{2†}	18.6 (2.1), 14.4–20.9	18.0 (2.1), 14.7–20.9	0.38
Cigarette smoking, pack years [†]	62.0 (30.9), 3.8–125	52.5 (28.8), 0.0–97.5	0.38
Pulmonary function [†]			
FEV1, L	0.78 (0.20), 0.54–1.21	0.77 (0.21), 0.47–1.21	0.90
%FEV1, % predicted	31.6 (8.1), 21.2–49.5	34.5 (9.1), 17.7–45.9	0.32
FEV1/FVC, %	38.0 (8.9), 24.6–50.5	38.8 (8.7), 25.4–52.9	0.74
VC, L	2.48 (0.37), 1.90–3.45	2.52 (0.50), 1.62–3.69	0.98
%VC, %	78.8 (9.3), 64.0–94.3	84.5 (12.6), 71.4–113.4	0.38
Exercise capacity on ICPET [†]			
Peak $\dot{V}O_2$, ml/kg/min	11.5 (3.3), 5.2–17.5	11.3 (3.5), 6.2–18.7	0.74
6-MWWD, m [†]	328 (110), 148–619	315 (118), 85–498	0.84
SGRQ [†]			
Total score	58.2 (16.5), 36.3–84.4	50.2 (15.5), 21.3–77.3	0.23
Symptoms score	61.5 (22.5), 29.4–97.5	51.6 (19.8), 19.7–78.5	0.34
Activity score	72.5 (14.9), 41.7–92.5	65.9 (16.3), 35.3–92.5	0.34
Impacts score	46.7 (19.5), 20.0–84.4	39.2 (17.7), 9.4–69.7	0.53
Medications [†]			
LAMA	9	6	0.27
SAMA	3	2	0.65
LABA	9	7	0.46
SABA	2	0	0.22
ICS	5	2	0.21
Methylxanthines	7	7	1.00

Data are presented as means (SD), and the minimum and maximum values unless otherwise stated. BMI = body mass index; FEV1 = forced expiratory volume in one second; FVC = forced vital capacity; ICPET = incremental cardiopulmonary exercise testing; ICS = inhaled corticosteroids; LABA = long-acting β_2 -agonist; LAMA = long-acting muscarinic antagonist; SABA = short-acting β_2 -agonist; SAMA = short-acting muscarinic antagonist; VC = vital capacity.

*The groups shown represent only patients analyzed for efficacy. Medications are not mutually exclusive, and data are presented separately.

[†]Analyzed using a Wilcoxon rank sum test.

[‡]Analyzed using a Fisher's exact test.

doi:10.1371/journal.pone.0035708.t001

Pulmonary Rehabilitation

Exercise training, which was included in the PR program, was conducted in three sets daily, every weekday for 3 weeks (i.e. 15 days) at high-intensity targets. Additional details are described online in Supplementary Methods S1.

Outcome Measure

Efficacy: The primary outcomes were changes in 6-min walk distance (6-MWD) and the score evaluated using the St. George Respiratory Questionnaire (SGRQ) [14]. Secondary outcomes were changes in the health-related QoL (HRQoL) score using the Short-Form 36 questionnaire (SF 36 v2™ Health Survey, Japanese version) [15,16,17] and the Medical Research Council (MRC) dyspnea scale [18], peak oxygen uptake ($\dot{V}O_2$), food intake, FEV1/FVC, vital capacity (VC), respiratory muscle strength, and plasma norepinephrine levels in the resting condition.

Safety: All randomized patients who received at least one dose of the study treatments (ghrelin group, n = 18; placebo group, n = 15) were included in the safety analyses using intention-to-treat analysis. Blood tests were done up to Week 7. All serious adverse events were monitored throughout the study period.

6-min Walk Test

The 6-MWD was measured as described previously [13].

Cardiopulmonary Exercise Testing (CPET)

While breathing room air with a mask, symptom-limited CPET was conducted on an electrically braked cycle ergometer using an incremental protocol (continuous ramp rate of 5 W/min). Expired gas data were measured breath-by-breath and collected as 30-s averages at rest and during exercise. The CPET was done until subject exhaustion.

Food Intake

Food intake was assessed as described previously [13].

Respiratory and Peripheral Muscle Strength

The maximal inspiratory pressure (MIP) and maximal expiratory pressure (MEP) were measured as described previously [13]. Peripheral muscle strength was measured by the maximal voluntary handgrip maneuver as described previously [13].

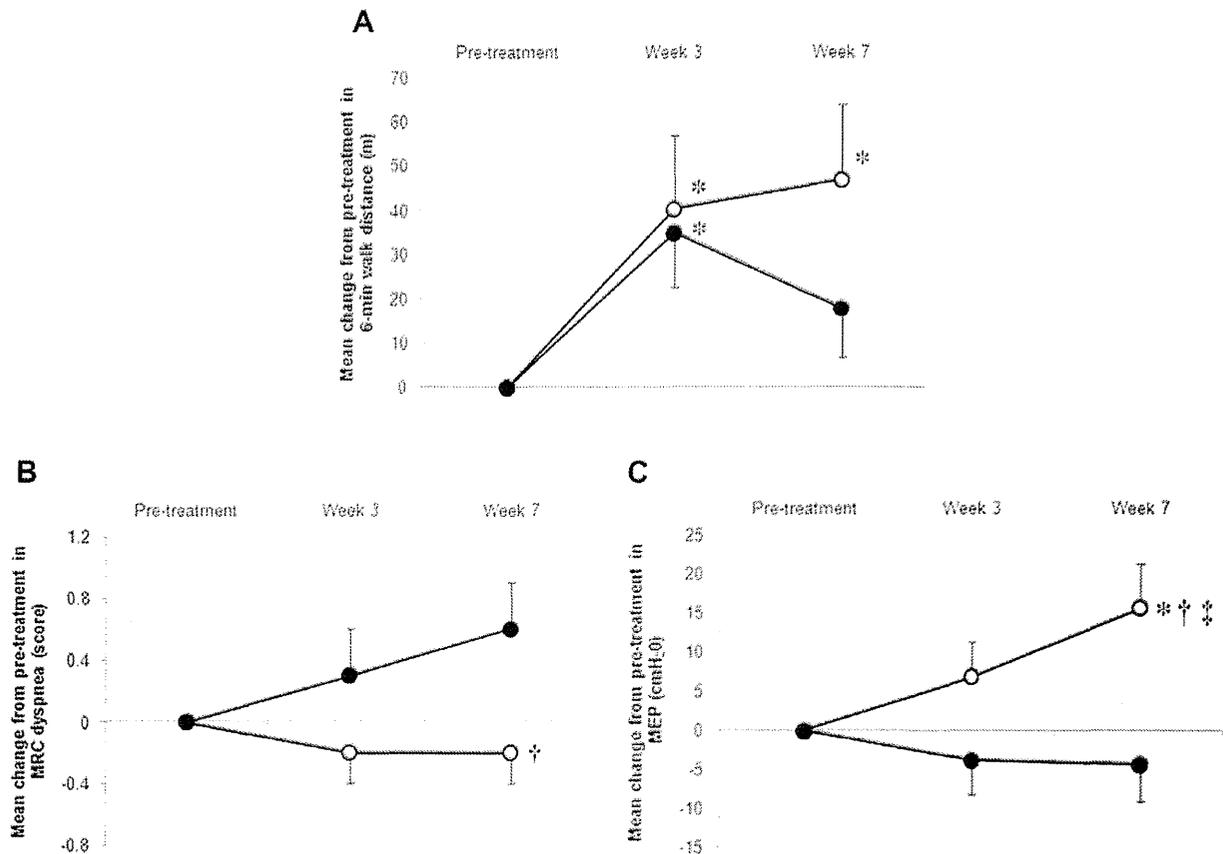


Figure 2. Change from pre-treatment in 6-min walk distance (6-MWD), Medical Research Council (MRC) score, and maximal expiratory pressure (MEP) over time. Open circles, ghrelin; closed circles, placebo. Data are presented as mean differences ± SE. * p < 0.05: change between pre- and post-treatment (within-group difference). † p < 0.05: change between pre-treatment and post-treatment (between ghrelin and placebo group difference). ‡ p < 0.05: time course effect of ghrelin versus placebo by repeated-measures ANOVA. A) In both groups, 6-MWD increases significantly to a similar level from pre-treatment at Week 3. Prolonged effects can be seen in the ghrelin group at Week 7, though the improvement in 6-MWD declined in the placebo group. B) Though the MRC score became progressively worse in the placebo group, the maintained effects in the MRC score can be seen in the ghrelin group at Week 7. C) Repeated-measures ANOVA indicated significant time course effects of ghrelin versus placebo in MEP (F (2, 51) = 4.17, p = 0.021). doi:10.1371/journal.pone.0035708.g002

Table 2. Changes in pre-treatment exercise capacity, pulmonary function and other parameters during pulmonary rehabilitation with ghrelin or placebo.

	At Week 3			At Week 7		
	Ghrelin, n = 14	Placebo, n = 15	Treatment effect (95% CI; p value)	Ghrelin, n = 14	Placebo, n = 13	Treatment effect (95% CI; p value)
Exercise capacity						
6-MWD, m	40 (17)*	35 (12)*	5 (−37 to 48; 0.81)	47 (17)*	18 (11)	29 (−15 to 73; 0.19)
Peak $\dot{V}O_2$, ml/min/kg	1.2 (0.4)*	0.5 (0.3)	0.7 (−0.4 to 1.8; 0.21)	ND	ND	ND
Peak $\dot{V}O_2$ /HR, ml/beats	0.5 (0.2)*	−0.4 (0.5)	0.9 (−0.2 to 2.0; 0.11)	ND	ND	ND
PFT						
FEV1/FVC, %	−1.1 (1.0)	−2.7 (0.9) [†]	1.6 (−1.2 to 4.3; 0.26)	−1.7 (1.2)	−1.2 (1.1)	−0.5 (−3.8 to 2.8; 0.77)
VC, L	0.14 (0.07)	0.11 (0.07)	0.03 (−0.16 to 0.23; 0.74)	0.09 (0.11)	−0.10 (0.07)	0.19 (−0.09 to 0.47; 0.17)
Others						
MIP, cmH ₂ O	−8.2 (4.9)	−9.8 (3.2)**	1.6 (−10.1 to 13.4; 0.78)	−8.4 (5.6)	−4.3 (2.6)	−4.1 (−17.7 to 9.5; 0.52)
MEP, cmH ₂ O	6.8 (4.4)	−3.8 (4.5)	10.7 (−2.2 to 23.5; 0.099)	15.6 (5.7) [†]	−4.3 (4.8)	19.9 (4.1 to 35.6; 0.015)
Food intake, kcal/day	122 (93)	−17 (86)	139 (−122 to 399; 0.28)	ND	ND	ND
MRC, score	−0.2 (0.2)	0.3 (0.3)	−0.4 (−1.2 to 0.3; 0.22)	−0.2 (0.2)	0.6 (0.3)	−0.7 (−1.4 to −0.1; 0.030)
Plasma NE, ng/ml	−0.063 (0.061)	−0.066 (0.067)	0.004 (−0.183 to 0.190; 0.97)	ND	ND	ND
IL-6 NE, pg/ml	1.52 (1.33)	0.08 (0.21)	1.44 (−1.35 to 4.22; 0.31)	ND	ND	ND
TNF- α , pg/ml	0.29 (0.15)	0.08 (0.06)	0.21 (−0.12 to 0.54; 0.21)	ND	ND	ND
Mean BP, mmHg	−13 (3)**	−3 (4)	−10 (−20 to 1; 0.061)	−2 (3)	4 (4)	−6 (−17 to 4; 0.20)
Body weight, kg	0.1 (0.3)	0.4 (0.3)	−0.3 (−1.2 to 0.7; 0.58)	0.8 (0.4)	0.4 (0.4)	0.4 (−0.7 to 1.4; 0.49)
Total lean mass, kg	0.2 (0.5)	0.5 (0.3)	−0.2 (−1.5 to 1.1; 0.73)	ND	ND	ND
Grip strength, kg	0.3 (0.9)	−0.0 (0.5)	0.3 (−1.7 to 2.3; 0.76)	1.1 (0.9)	2.5 (1.1)*	−1.5 (−4.4 to 1.4; 0.31)

Data are means (SE), or mean effect (95% CI; p value) unless otherwise indicated. BP = blood pressure; FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity; IL = interleukin; MEP = maximal expiratory pressure; MIP = maximal inspiratory pressure; MRC = medical research council; ND = not done; NE = norepinephrine; PFT = pulmonary function test; VC = vital capacity.

*p<0.05,

**p<0.01: change between pre-treatment and post-treatment within-group difference.

doi:10.1371/journal.pone.0035708.t002

Dual-Energy X-ray Absorptiometry (DEXA)

All participating centers measured dual energy x-ray absorptiometry (DEXA) to assess the total body composition, including lean body mass. The measurements were performed with the subject lying in a supine position. As a general rule, a single expert from each center analyzed the scans from the corresponding center.

Blood Samples and Analyses

Serum GH, serum insulin-like growth factor (IGF)-1, serum tumor necrosis factor α (TNF- α), serum interleukin-6 (IL-6), and plasma norepinephrine were measured as described previously [13]. Additional details are described online in Supplementary Methods S1.

Sample Size

The study's target accrual was 60 in the original protocol at the time of study design (see supporting information; Protocol S1). When 31 of the 33 randomized patients completed this study, we re-performed the power and sample size calculation, and confirmed that the number of patients that had completed the study exceeded the number necessary for the re-calculated sample size of 18. As a result, this trial ended prematurely. Because i) it is difficult to prolong hospitalization considering the current status of

health care insurance in Japan, and ii) what constituted a clinically important change in 6-MWD after ghrelin treatment with PR was not known before the study ended; the sample size calculation was re-performed on the estimated effect of only ghrelin treatment for improving 6-MWD, which was based on information from the pilot study [13]. The resultant total sample size of 18 was finally used to provide the power (80%) to detect a mean difference of 60 m in 6-MWD with an estimated SD of 40 m using a two-sided alpha of 0.05, though the study's target accrual stated in the original protocol was 60.

Statistical Analysis

All data are expressed as means \pm SD or SE unless otherwise indicated. Comparisons of baseline characteristics between the two groups were made by Fisher's exact tests and Wilcoxon rank sum tests. Effects were examined once or twice; that is i) at Week 3 soon after 3-week treatment or ii) at Week 3 and Week 7 (i.e., 4 weeks after the completion of 3-week treatment). The results at Week 3 and Week 7, respectively, were compared with the pre-treatment within each group, and between the two groups using paired *t*-tests and unpaired *t*-tests, respectively. To assess the time course efficacy of ghrelin versus placebo, post-treatment data up to Week 7 were also assessed using a repeated-measures analysis of variance

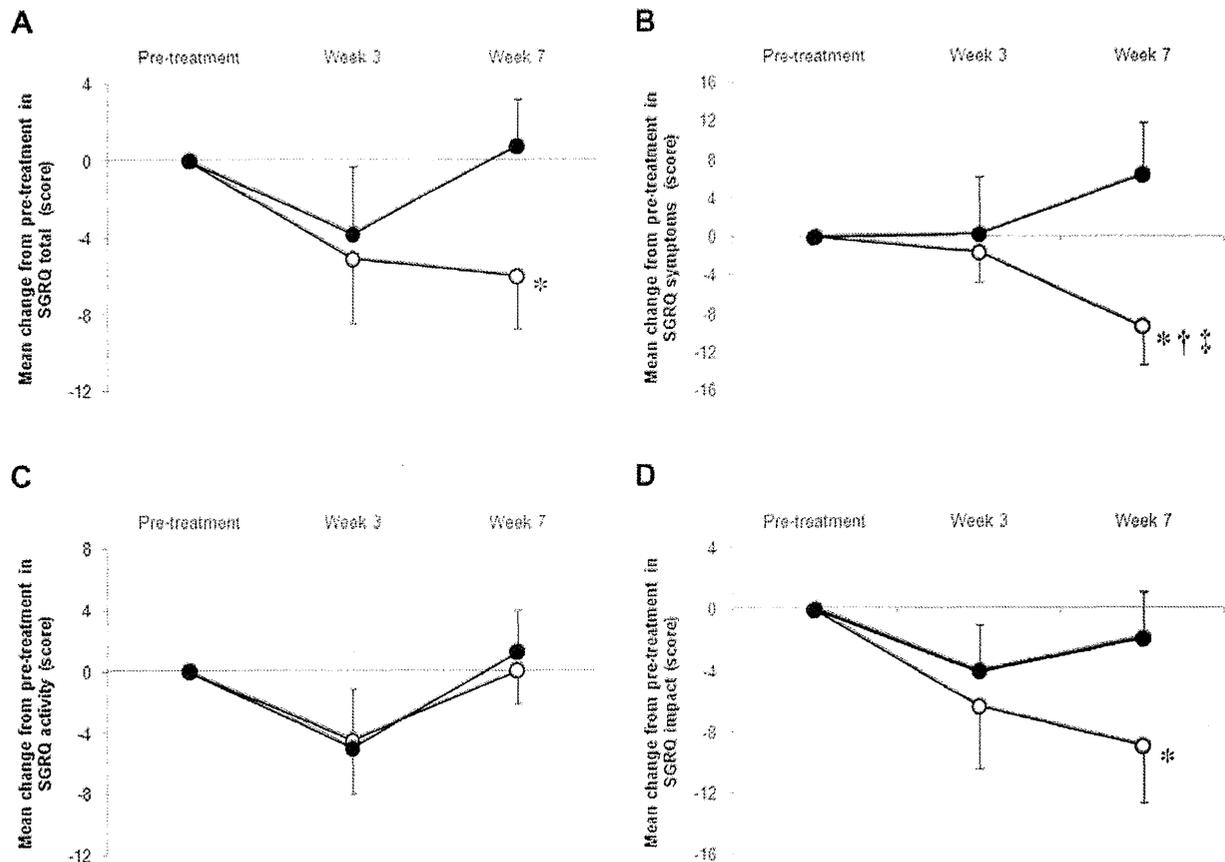


Figure 3. Change from pre-treatment in St. George Respiratory Questionnaire (SGRQ) scores over time. Open circles, ghrelin; closed circles, placebo. Data are presented as mean differences \pm SE. * $p < 0.05$: change between pre- and post-treatment (within-group difference). † $p < 0.05$: change between pre-treatment and post-treatment (between ghrelin and placebo group difference). ‡ $p < 0.05$: time course effect of ghrelin versus placebo by repeated-measures ANOVA. At Week 3, marked improvements in SGRQ scores are not seen in both groups. However, SGRQ scores, especially SGRQ symptom scores, are significantly improved in the ghrelin group at Week 7. B) Repeated-measures ANOVA indicated significant time course effects of ghrelin versus placebo in SGRQ symptoms ($F(2, 51) = 3.19$, $p = 0.049$). doi:10.1371/journal.pone.0035708.g003

(ANOVA). A p value < 0.05 was considered significant (SAS 9.1.3, SAS Institute Inc., Cary, NC, USA).

Results

Of the 33 randomized patients, 31 completed the 3-week study; 2 patients in the ghrelin group discontinued study medications due to pneumonia and depression, respectively. Of the 31 patients who completed the randomized 3-week study, in the ghrelin group, one patient had infective enteritis after 3 weeks of medications, and one had low back pain due to lumbar spondylosis before and throughout the 3 weeks of medications. Two patients in the placebo group were lost to follow-up after the Week 3 measurements. Therefore, 29 patients (ghrelin, $n = 14$; placebo, $n = 15$) were included in the study analyses to ensure adequate efficacy evaluation using pre-protocol analysis. The mean BMI in the enrolled patients ($n = 29$) was very low (mean \pm SD, 18.3 ± 2.1 kg/m²). The treatment groups were generally well-matched with regard to demographics and baseline characteristics (Table 1).

Somatotropic Function

At pre-treatment, compared with placebo, a single administration of ghrelin markedly increased serum GH levels from baseline (mean change \pm SE: ghrelin group 46.4 ± 6.2 ng/ml at the mean peak time (35 min) versus the placebo group 1.1 ± 0.5 ng/ml at the mean peak time (55 min); between group $p < 0.0001$), the effect of which was maintained at Week 3 (mean change \pm SE: ghrelin group 15.8 ± 2.1 ng/ml at the mean peak time (30 min) versus the placebo group 0.4 ± 0.2 ng/ml at the mean peak time (65 min); between group $p < 0.0001$). Three-week ghrelin-PR combination treatment tended to increase serum IGF-1 levels (mean change \pm SE: 12 ± 6 ng/ml, within-group $p = 0.093$).

Exercise Tolerance and Gas Exchange Measurements

At both Week 3 and Week 7, there were no significant differences between the ghrelin and placebo groups in 6-MWD. In each group, at Week 3, a similar significant increase from pre-treatment in 6-MWD was observed (mean difference: ghrelin group +40 m, within group $p = 0.033$ versus placebo group +35 m, within group $p = 0.013$). The effect remained at Week 7 in the ghrelin group, whereas in the placebo group, the

Table 3. Changes in pre-treatment scores of health-related quality of life during pulmonary rehabilitation with ghrelin or placebo

	At Week 3			At Week 7		
	Ghrelin, n = 14	Placebo, n = 15	Treatment effect (95% CI; p value)	Ghrelin, n = 14	Placebo, n = 13	Treatment effect (95% CI; p value)
SGRQ						
Total	-5.0 (3.2)	-3.9 (3.5)	-1.1 (-10.9 to 8.7; 0.83)	-6.0 (2.7)*	0.8 (2.4)	-6.8 (-14.4 to 0.7; 0.072)
Symptoms	-1.7 (3.0)	0.3 (5.9)	-1.9 (-16.2 to 12.3; 0.77)	-9.4 (4.0)*	6.4 (5.4)	-15.8 (-29.5 to -2.1; 0.026)
Activity	-4.5 (3.5)	-5.0 (3.9)	0.4 (-10.5 to 11.4; 0.94)	0.1 (2.2)	1.3 (2.7)	-1.2 (-8.3 to 5.9; 0.73)
Impacts	-6.3 (4.1)	-4.1 (3.1)	-2.2 (-12.6 to 8.2; 0.67)	-8.9 (3.7)*	-1.9 (3.0)	-7.0 (-16.9 to 2.9; 0.16)
SF-36						
Physical functioning	4.6 (6.1)	0.3 (3.9)	4.3 (-10.0 to 18.5; 0.55)	3.1 (4.7)	-6.9 (4.9)	10.0 (-3.9 to 23.9; 0.15)
Role physical	-8.3 (6.9)	-4.6 (5.4)	-3.7 (-21.6 to 14.1; 0.67)	-12.0 (4.1)*	-22.6 (7.3)**	10.6 (-6.8 to 27.9; 0.22)
Bodily pain	-6.8 (5.3)	8.4 (6.4)	-15.2 (-33.0 to 2.6; 0.090)	-7.6 (6.5)	-3.8 (6.8)	-3.8 (-23.2 to 15.7; 0.69)
General health	-0.6 (4.5)	2.9 (5.2)	-3.5 (-17.9 to 11.0; 0.63)	0.5 (3.4)	5.8 (5.4)	-5.3 (-18.5 to 7.9; 0.41)
Vitality	5.7 (5.5)	7.8 (4.4)	-2.0 (-16.3 to 12.3; 0.77)	3.4 (4.8)	-2.9 (3.4)	6.2 (-5.9 to 18.4; 0.30)
Social functioning	-3.1 (9.5)	3.3 (7.2)	-6.5 (-30.5 to 17.6; 0.59)	-12.5 (8.1)	-2.9 (6.0)	-9.6 (-30.5 to 11.3; 0.35)
Role emotional	-13.9 (5.2)*	-9.5 (9.2)	-4.4 (-27.7 to 18.8; 0.68)	-19.9 (6.6)*	-16.0 (10.4)	-3.9 (-29.3 to 21.5; 0.76)
Mental health	0.4 (6.0)	3.7 (4.2)	-3.3 (-18.0 to 11.5; 0.65)	3.5 (3.3)	-8.2 (4.6)	11.7 (0.0 to 23.4; 0.050)

Data are means (SE), or mean effect (95% CI; p value) unless otherwise indicated. SGRQ = St. George Respiratory Questionnaire; SF 36 = short-Form 36.

*p<0.05,

**p<0.01: change between pre-treatment and post-treatment within-group difference.

doi:10.1371/journal.pone.0035708.t003

improvement in 6-MWD was reduced at Week 7 (mean difference: ghrelin group within group +47 m, $p = 0.017$ versus placebo group +18 m, within group $p = 0.14$) (Table 2 and Figure 2A). To assess the time course efficacy of ghrelin versus placebo in 6-MWD, a repeated-measures ANOVA was performed. There was no significant time course effect of ghrelin versus placebo in 6-MWD ($F(2, 51) = 1.10$, $p = 0.34$).

In the ghrelin group, the peak $\dot{V}O_2$ and $\dot{V}O_2/HR$ were significantly increased by 1.2 ml/kg/min and 0.5 ml/beats, respectively, from pre-treatment (within-group $p = 0.021$, $p = 0.019$, respectively) (Table 2). However, there was no significant difference between the two groups in the peak $\dot{V}O_2$ and $\dot{V}O_2/HR$. In the ghrelin group, the ventilatory equivalents for oxygen ($\dot{V}E/\dot{V}O_2$) was relatively improved by -3.9 from pre-treatment (within group $p = 0.060$).

Table 4. Adverse events.

Event	Ghrelin, n = 18	Placebo, n = 15
Patients with at least 1 adverse event	12 (67)	5 (33)
Adverse events not considered study therapy-related		
Pneumonia	1 (6)	0 (0)
Depression	1 (6)	0 (0)
Infective enteritis	1 (6)	0 (0)
Lung cancer*	1 (6)	0 (0)
Hypercalcemia	0 (0)	1 (7)
Adverse events considered study therapy-related		
Stomach rumbling	3 (17)	2 (13)
Feeling of being warm	4 (22)	0 (0)
Feeling of hunger	2 (11)	2 (13)
Thirst	2 (11)	0 (0)
Slight liver dysfunction	1 (6)	0 (0)
Hypercholesterolemia	1 (6)	0 (0)
Hypoproteinemia	1 (6)	2 (13)

Values are presented as n (% of group). * One patient developed lung cancer 2 years and 9 months after study treatment.

doi:10.1371/journal.pone.0035708.t004

HRQoL and MRC Measures

In both groups, there was no significant difference in each SGRQ score and MRC score between pre-treatment and at Week 3. At Week 7, there was a significant treatment effect between the two groups in SGRQ symptoms (between-group: $p = 0.026$, Table 3 and Figure 3B), and in the MRC score (between-group $p = 0.030$, Table 2 and Figure 2B). At Week 7, in the ghrelin group, SGRQ total was decreased by 6.0 from pre-treatment (within-group $p = 0.046$, between-group $p = 0.072$) (Table 3 and Figure 3A). Furthermore, there was a significant time course effect of ghrelin versus placebo in SGRQ symptoms (repeated-measures ANOVA, $F(2, 51) = 3.19$, $p = 0.049$, Figure 3B).

Body Weight and Food Intake

In the ghrelin group, at Week 1, the relative increase in body weight was +0.42 kg (within group $p = 0.092$), which was reduced by Week 3 and followed by a re-increase at Week 7 (+0.8 kg, within group: $p = 0.054$). However there was no significant difference in body weight between the groups at each Week (Table 2). No affect on whole lean body mass from ghrelin was seen at Week 3 (Table 2). No significant increase from baseline in food intake was observed at Week 3 in both groups (Table 2).

Respiratory and Peripheral Muscle Strength

In the ghrelin group, at Week 3, the post-treatment increase in respiratory muscle strength, as indicated by MEP and MIP, was not significantly different from that in the placebo group, but at Week 7, the mean increase from pre-treatment in MEP (+15.6 cmH₂O) was significantly different from that in the placebo group (between group $p=0.015$) (Table 2). Furthermore, there was a significant time course effect of ghrelin versus placebo in MEP (repeated-measures ANOVA, $F(2, 51)=4.17$, $p=0.021$, Figure 2C).

At Week 3 and Week 7, there was no significant treatment effect between the two groups in grip strength (Table 2).

Pulmonary Function, Plasma Norepinephrine, and Other Hormone Levels

Ghrelin treatment did not significantly change any parameters of the pulmonary function tests, serum TNF- α , serum IL-6, or plasma norepinephrine at rest (Table 2).

Safety

Throughout this trial, 67% of patients in the ghrelin group and 33% of patients in the placebo group reported 12 and 5 adverse events, respectively, but there was no significant difference between the groups (Table 4). In the ghrelin group, alanine aminotransferase increased to 41 IU/L in one patient (6%), and total cholesterol increased to 270 mg/dl in one patient (6%); both increases disappeared at Week 7. Two patients randomized to ghrelin discontinued as a result of adverse events: one because of bacterial pneumonia, and one because of depression, both of which were not considered related to ghrelin treatment. One patient randomized to ghrelin developed lung cancer 2 years and 9 months after the end of ghrelin administration, but this was judged by the efficacy and safety committee as not causally related to ghrelin treatment, considering the period of disease development and the incidence rate of lung cancer [19].

Discussion

The present study is the first multicenter, randomized, double-blind, placebo-controlled study to assess the effect and safety of repeated ghrelin administration to very severe cachectic patients with COPD. The main results of this study can be summarized as follows. In the ghrelin group, single administration of ghrelin was accompanied by a significant increase in serum GH levels during 3-week treatment, and there was no significant difference in 6-MWD between ghrelin and placebo at Week 3 and at Week 7. With ghrelin, symptomatic improvements in SGRQ symptoms and MRC score were not obtained at Week 3, but significant differences between ghrelin and placebo were seen at Week 7. In the ghrelin group, no significant within-group improvement from pre-treatment was seen in respiratory muscle strength, as indicated by MEP and MIP, at Week 3, but there was a significant difference in MEP between ghrelin and placebo at Week 7. Repeated-measures ANOVA showed significant time course effects of ghrelin versus placebo in SGRQ symptoms and MEP. Finally, ghrelin treatment was well tolerated.

Ghrelin treatment may have beneficial, continuing effects after treatment on HRQoL and MRC measures in this population. Though this study was conducted to determine the effectiveness of ghrelin in cachectic COPD patients, considering a synergistic interaction between ghrelin and PR, the data of this study need to be interpreted with caution, because, especially in advanced stage patients, excessive exercise training may partially worsen the anabolic and catabolic balance [1,20]. In the present study, which

included patients with a lower exercise capacity and pulmonary function than those in the pilot study [13] and more cachectic patients than those in other studies on PR [21], the 6-MWD after 3-week PR in the placebo group was decreased in 3 (20%) of the 15 patients. Since 5 patients (33%) in the placebo group found the initial training work rate intolerable, the initial training work rate remained at its initial setting. In addition, at Week 3, outcome measurements showed no improvements with ghrelin compared with placebo. These findings may represent patients' variable responses to PR, which might have an influence on the effects of ghrelin. Of note, however, there were significant treatment effects of ghrelin in both SGRQ symptoms and MRC score. In addition, the treatment tended to improve the total SGRQ score by more than 4 points; a clinically meaningful improvement. These effects were not observed soon after the 3 week-treatment, but were seen 4 weeks after treatment, maintaining the improvement obtained in 6-MWD at Week 3. Similarly, 4 weeks after treatment, the effect of ghrelin on respiratory muscle strength was confirmed, though it has been reported that GH alone does not increase strength in healthy elderly [22,23,24]. Furthermore, repeated-measures ANOVA indicated significant time course effects of ghrelin versus placebo in SGRQ symptoms and MEP. Our data suggest that improving of the respiratory muscle strength, the O₂ pulse, and the ventilatory equivalents for oxygen may serve as a mechanism by which ghrelin-PR combination treatment improved symptoms, though further examination is needed to understand the precise mechanism. These findings suggest that repeated ghrelin administration may have beneficial, sustained effects after administration on symptoms through GH-dependent and/or -independent mechanisms.

Cachectic elderly patients with COPD who were given intravenous ghrelin showed a continuous increase of pulsatile GH secretion in the present study. There is evidence that insufficiency of sarcopenia-related hormones, such as GH and IGF-1, may contribute to cachexia [25,26]. Observational studies in cachectic COPD patients have found decreased levels of these hormones [27,28]. In the present study, despite significant increases in GH secretion levels throughout the 3-week treatment and respiratory muscle strength, ghrelin provided only a significant within-group increase in exercise performance, and a relative within-group increase in IGF-1 levels and body weight. Furthermore, ghrelin did not affect food intake, grip strength or plasma norepinephrine levels at rest in the present study. Although DEXA should be performed a greater number of times during the trial, at Week 3 ghrelin did not show any effects on whole lean body mass. Meanwhile, previous studies showed that ghrelin administration induced a positive energy balance and weight gain [8], increased food intake [9,13], and decreased sympathetic nervous activity [10,11,13]. The discrepancy may be explained by the fact that the intensity of exercise training for some cachectic participants counteracted the effects of ghrelin, though lower extremity exercise training at higher intensity produces greater benefits than lower intensity training [4]. As one of the reasons, the patients treated with both ghrelin and exercise training gained at Week 1, which was not seen in the placebo group. However, this weight gain reduced by Week 3. At Week 7, the weight was regained (Table 2). The days of attending PR in the ghrelin group was negatively correlated with the increase in body weight from Week 3 to Week 7 ($r=-0.710$, $p=0.003$). We speculate that the unintended excessive exercise permitted by ghrelin administration with antidepressant-like effects [29] might prevent the obtained results. Nevertheless, these findings suggest that clinical interventions with ghrelin may help cachectic COPD patients via inhibiting somatopause and regulating metabolic balance.