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Case Report

Autopsy case of primary myelofibrosis in which myeloid sarcoma was the initial manifestation of tumor progression

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Myeloid sarcoma (MyS) is defined as an extramedullary tumor-forming neoplasm consisting of immature myeloid cells with/without maturation. We experienced a case involving a 68-year-old Japanese male patient who had been followed-up for four years with a diagnosis of chronic idiopathic myelofibrosis/primary myelofibrosis (PMF) and noticed a painful mass in his left axilla. A wedge biopsy characterized the lesion as MyS that displayed megakaryoblastic/megakaryocytic differentiation. As his complete blood count included a few myeloid blasts (1% of WBC) and a bone marrow biopsy detected fibrosis without evidence of acute myelogenous leukemia (AML), a diagnosis of extramedullary blastic transformation of PMF was made, which was confirmed later by V617F mutation in *Janus kinase*-2 in both initial bone marrow biopsy and axillary tumor biopsy specimens. The patient died of pneumonia eight months after developing the axillary tumor. At autopsy, multiple MyS masses were detected in his soft tissue, but his bone marrow only contained fibrosis. Although MyS rarely develops before the leukemic transformation of PMF, no evidence of AML could be found in the patient's bone marrow at any point during the course of his disease. Thus, it is possible that the blasts in his peripheral blood were derived from the remaining MyS. Furthermore, the present case indicates that extramedullary blastic transformation, which is occasionally seen in CML, can also occur in PMF. Therefore, it is important to recognize that there is a wide variation in the pathogeneses of MyS and PMF.

Key words: autopsy, megakaryocytic differentiation, myeloid sarcoma, primary myelofibrosis

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Myeloid sarcoma (MyS), which was formerly referred to as chloroma, granulocytic sarcoma, or extramedullary myeloid tumor, is defined as an extramedullary tumor-forming neoplasm consisting of immature myeloid cells with/without maturation.[1] MyS can be hematopathologically subclassified into either granulocytic sarcoma, monoblastic sarcoma, megakaryoblastic sarcoma, or rarely, erythroblastic sarcoma, based on the predominant proliferative component. Myeloid sarcoma can occur as the first sign of a blast crisis in chronic myelogenous leukemia (CML) and is included in the diagnostic criteria for blast crisis in CML. However, a similar pathologic condition can also occur in myeloproliferative neoplasms (MPN)/chronic myeloproliferative disorders other than CML.

The recent discovery of a recurrent V617F mutation in *Janus kinase*-2 (*JAK*-2)[2] has brought about considerable progress in the understanding of the pathogenesis of MPN other than CML, and it is present in most of patients with polycythemia vera and in approximately a half of patients with essential thrombocythemia or primary myelofibrosis (PMF)/chronic idiopathic myelofibrosis.[2–4] Furthermore, the discovery has improved the diagnostic accuracy, because this point mutation could be detected using DNA samples from paraffin-embedded pathologic specimens.[5]

Recently, we experienced a case of MyS that developed during the course of PMF. Polymerase chain reaction (PCR) amplification could show *JAK*-2 mutation in both a bone marrow biopsy specimen obtained at the time of PMF and a tissue containing MyS. In the present report, we describe the hematopathological features of the case together with a review of the literature. The purpose of this report is to indicate that blast crises could develop not only in CML, but also in PMF.

CASE REPORT

A 64-year-old Japanese man was first admitted to our hospital because of general malaise. On physical examination,

Table 1 Peripheral blood findings

	Initial presentation	Tumor formation†	Blastic crisis	Death
Clinical course (month)	0	39	46	48
RBC (x10⁴/μL)	399	321	270	224
Hb (g/dL)	12.0	8.6	8.3	6.3
WBC (/μL)	5400	4600	6100	9800
Neutrophil (%)	73.0	72.0	75.0	6.0
Lymphocyte (%)	24.0	17.0	2.0	1.0
Blast (%)	0.0	1.0	21.0	91.0
Platelet (x10⁴/μL)	19.6	17.4	13.8	1.7
BM finding	PMF	PMF	ND	PMF

†At the time of tumor formation
ND, not done; PMF, primary myelofibrosis.

his spleen and liver were impalpable, and no peripheral lymphadenopathy was detected. His complete blood count (CBC) revealed a borderline degree of anemia (hemoglobin concentration: 12.0 g/L; red blood cell (RBC) count: 3.99 × 10^12/L), but his platelet and white blood cell (WBC) counts were within normal limits and no abnormal cells were detected on peripheral blood (PB) films. As bone marrow aspiration was unsuccessful despite several attempts, no chromosomal analysis was performed. Bone marrow biopsy revealed the proliferation of abnormal megakaryocytes with severe reticulin fibrosis (Fig. 1). The patient was diagnosed as having chronic idiopathic myelofibrosis based on the criteria given by the WHO classification-2001[6] at that time.

Four years later (at the age of 68 years), a painful mass was noticed in his left axilla. The lesion was histologically characterized as MyS with megakaryoblastic/megakaryocytic differentiation by a wedge biopsy (Fig. 2). At this time, his CBC included a hemoglobin level of 8.6 g/L, an RBC count of 3.21 × 10^12/L, and a WBC count of 4.6 × 10^9/L with 1% myeloid blasts. A bone marrow biopsy revealed fibrosis without evidence of acute myelogenous leukemia (AML). However, an increased number of myeloid blasts were noted in his PB, and they accounted for 78% of WBC at one month after the biopsy of the axillary tumor. Combination chemotherapy consisting of cytosine arabinoside (10 mg/m^2) and aclarubicin (14 mg/m^2), and local radiotherapy, resulted in reductions in the size of the tumor and the number of myeloid blasts in his PB. However, the tumor enlarged rapidly and the number of myeloid blasts in his PB had increased to 78% of WBC within a month (Table 1). The patient died of pneumonia at 8 months after developing the axillary tumor, and an autopsy was performed.

HEMATOPATHOLOGICAL FEATURES

The first bone marrow biopsy

The marrow space was diffusely occupied by fibrous tissue, which was positively stained by silver reticulin stain. The number of normal hematopoietic cells was decreased, but clusters of megakaryoblastoid cells were noted. The specimen was reviewed at the time of biopsy of the left axillary tumor and fibrosis was graded as either 1 or 2.[7]

Biopsy of the left axillary tumor

Dense diffuse growth of large mononuclear cells was noted among the patient's subcutaneous adipose tissue and striated muscle, and the lesion was associated with diffuse fibrosis. The proliferating cells had prominent single nuclei and relatively abundant amphophilic cytoplasm. The number of cells in mitosis was increased, and numerous apoptotic bodies were detected. In addition, giant cells resembling megakaryocytes were occasionally seen.

These cells were immunohistochemically examined using the avidin-biotin-peroxidase complex method and antibodies against cytoplasmic CD3 (cCD3; Dako Japan, Tokyo, Japan), CD10 (Leica Microsystems, Tokyo, Japan), CD20 (Dako Japan), CD34 (Dako Japan), CD42b (Nihon Millipore, Tokyo, Japan), CD43 (Dako Japan), CD68 (Leica Microsystems), CD79a (Dako Japan), α1-antichimotrypsin (AACT; Dako Japan), myeloperoxidase (MPO; Dako Japan), von Willebrand factor (vWF; Dako Japan), and glycophorin A (Dako Japan). As a result, we found that the majority of proliferating cells were positive for CD34 and CD43, and some of them were positive for AACT. The giant cells were positive for CD42b, but the proliferating cells were negative for CD3, CD10, CD20, CD68, CD79a, MPO, vWF, and glycophorin A. Based on these findings, the tumor was diagnosed as MyS with megakaryoblastic/megakaryocytic differentiation.

The second bone marrow biopsy

This was performed at the time of the biopsy of the axillary tumor. The histopathologic features of the biopsy sample were similar to those of the first biopsy, although an immunohistochemical examination revealed the occasional

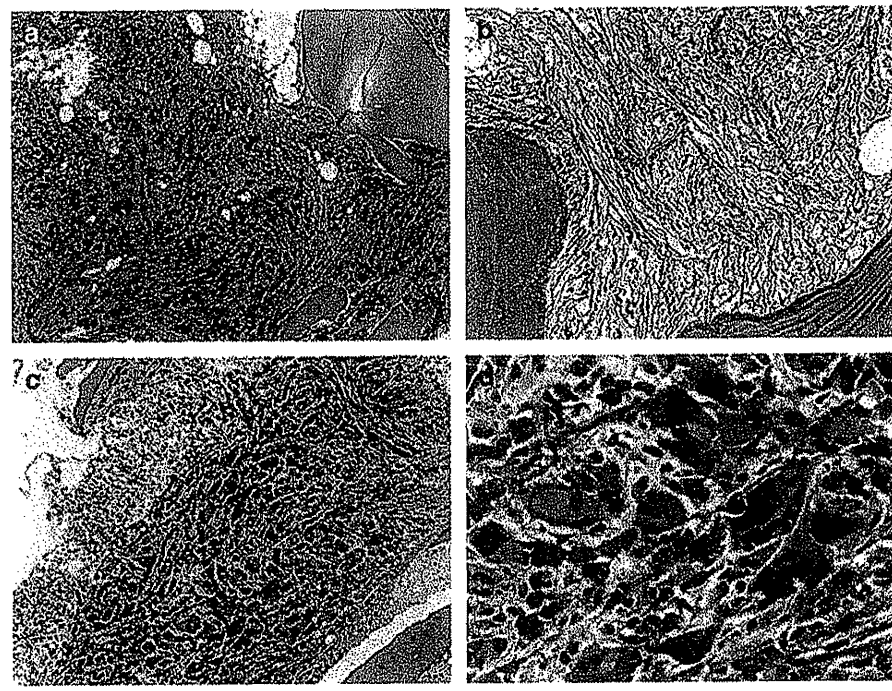

Figure 1 Bone marrow features at the first admission (**a, b**) and autopsy (**c, d**). The marrow space is subtotally occupied by cellular and fibrous growth without features of acute myeloid leukemia (AML). (a, H&E, x10). Prominent reticulin fibrosis is evident (b, silver reticulin stain, x10). At autopsy, proliferation of giant cells with abundant eosinophilic cytoplasm is noted in the fibrous background (c, H&E, x10). (d) The majority of the proliferating cells are characterized as megakaryocytes with atypical nuclei, but no features of conventional AML are present (H&E, x40).

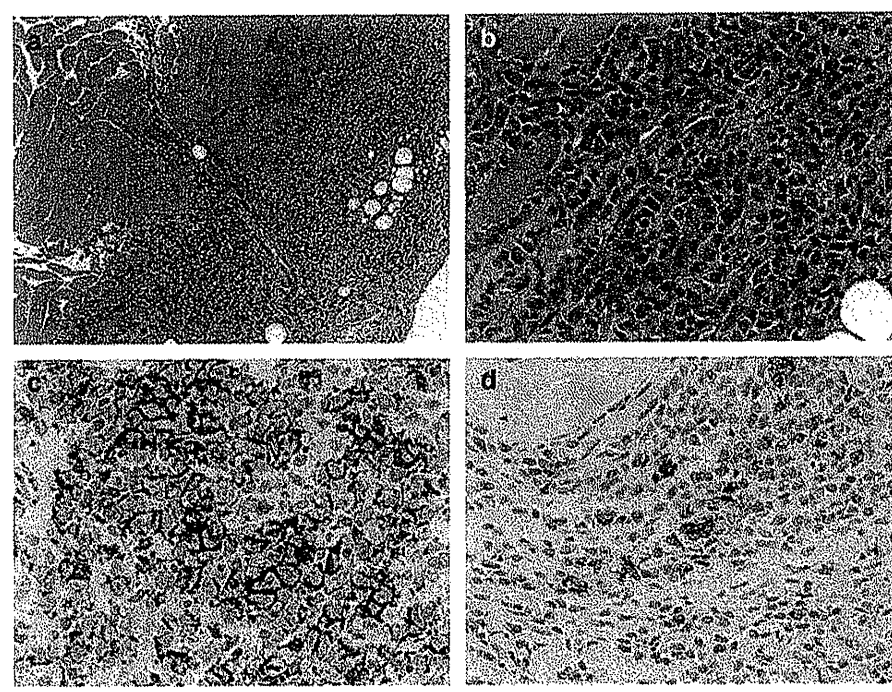

Figure 2 Histologic and immunohistochemical features of the left axillary tumor. Diffuse proliferation of mononuclear cells infiltrating into the striated muscle tissue is seen (**a**, H&E, x10). At the higher magnification, single-file pattern of growth of the cells is seen (**b**, H&E, x40). Immunohistochemically, the cells are positive for CD43 (**c**) and CD42b (**d**) (c and d, immunoperoxidase stain with hematoxylin counterstain; c ×40, d ×40).

presence of large cells that were positive for CD42b and von Willebrand factor.

Detection of the *JAK2* V617F mutation

Presence or absence of *JAK2* mutation was examined using DNA samples prepared form the paraffin embedded tissues of the first bone marrow biopsy and axillary tumor biopsy. Polymerase chain reaction (PCR) amplification was performed using the JAK2 MutaScreen™ Kit (IPSOGEN, Marseille, France) and amplicons were analyzed. Consequently, PCR amplification could show *JAK*-2 mutation in both samples (Fig. 3).

Autopsy

Multiple tissue samples from the vertebrae showed broad reticulin fibrosis with occasional collagenous fibrosis, which

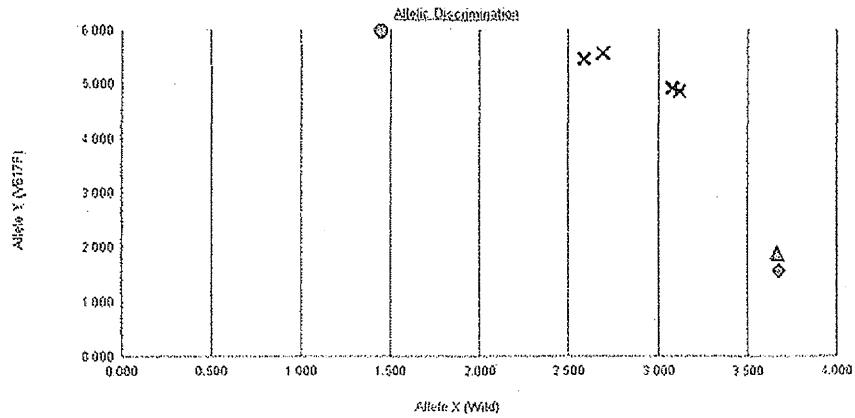

Figure 3 Scatter plot of the PCR results by JAK2 Muta*Screen*™ Kit. Fluorescent values of wild type DNA are plotted on the *x* axis, while those of DNA containing V617F on the *y* axis. Fluorescent values of both DNA samples (the initial bone marrow biopsy and axillary tumor biopsy) in duplicate are located in the upper light, indicating that one of the alleles in both DNA samples contains V617F.

Table 2 Reported cases of myeloid sarcoma developed before leukemic transformation

Reference number	Sex	Age(year)	Location of tumor	Period†	Diagnosis
8	F	22	Submandibular region	Two month	Granulocytic sarcoma
9	M	49	Duodenum	ND	Granulocytic sarcoma
10	M	56	Right inguinal (soft tissue)	Three month	Megakaryoblastic sarcoma
11	F	43	Inguinal lymph node	ND	Megakaryoblastic sarcoma
12	F	54	Face (soft tissue)	ND	Granulocytic sarcoma
This case	M	64	Left axilla	Seven month	Megakaryoblastic sarcoma

†A period from myeloid sarcoma to development of leukemic transformation.
ND, not described.

were confirmed by the corresponding histochemical staining examinations. Although a small number of normal hematopoietic cells including megakaryocytes remained, no foci that were indicative of blastic transformation were detected. These features were compatible with grade 2 PMF and were confirmed to be similar to those of the first and second bone marrow biopsies. No solid tumor was found in the left axillary region or at any other site. His spleen was enlarged (414 g) and displayed diffuse blastic cell proliferation in the red pulp, resulting in atrophy of the white pulp. The proliferation and/or infiltration of blastic cells without tumor formation were also seen in the esophagus, right kidney, urinary bladder, testes, and right lung (a tumor thrombus but not parenchymatous involvement).

DISCUSSION

In this case, a left axillary tumor appeared during the course of PMF, and biopsy revealed the diffuse infiltration of blastic cells with occasional cells having megakaryoblastic/megakaryocytic features. Although the lesion could be characterized as one of extramedullary tumor-forming AML or myeloid sarcoma with megakaryoblastic/megakaryocytic differentiation without other information, the lesion could be characterized as extramedullary blastic transformation of PMF, because of a history of PMF, the presence of only a few blasts in the peripheral blood and histologic features of the bone marrow (compatible with chronic phase PMF) at the time of the development of the axillary tumor, and the presence of *JAK2* mutation in both the initial bone marrow specimen and the axillary tumor. Although this interpretation seemed to be confirmed by the rapid increase in the number of blasts in his peripheral blood following the tumor biopsy, autopsy did not detect blastic transformation in the bone marrow. Thus, it is reasonable to consider that the blasts in his peripheral blood at the time of the rapid increase in their number were derived from the remaining axillary tumor, not from the bone marrow, although only vertebral bone marrow could be examined at autopsy.

Five cases of PMF in which MyS developed before leukemic transformation have been reported in the literature,[8–12]

and two of them were characterized as megakaryoblastic sarcoma[10,11] (Table 2). Leukemic transformation occurs in up to 50% of PMF,[7,13,14] but megakaryoblastic transformation is rare.[11] In our case, megakaryoblastic/megakaryocytic differentiation was only found in some parts of the tumor, while the majority of the cells remaining showed the morphological and immunohistochemical features of blasts without any phenotypically identifiable commitment to differentiation. Based on these findings, our case could be interpreted as blastic MyS with partial megakaryoblastic/megakaryocytic differentiation rather than megakaryoblastic sarcoma. Among the 92 cases of MyS reported by Pileri *et al.*,[15] 46 (50%) were characterized as belonging to the blastoid subtype, and only one case showed megakaryoblastic/megakaryocytic differentiation. Of these 92 cases, PMF had been diagnosed before the development of MyS in three cases and PMF was found at the time of MyS development in one case. On the other hand, 25 cases of MyS were not associated with any other myeloproliferative disorder, but two of these patients later developed AML. Among the 61 cases of PMF reported by Richard *et al.*,[16] three patients (5%) later developed MyS. Thus, it is indicated that both MyS with megakaryoblastic/megakaryocytic differentiation and the development of MyS during the course of PMF are rare phenomena.

The development of MyS before the leukemic transformation of PMF is a rare phenomenon, but it has been indicated that such MyS are more likely to be misdiagnosed, e.g., as malignant lymphoma or poorly differentiated sarcoma.[15] Furthermore, the axillary tumor itself could be diagnosis as MyS with megakaryoblastic/megakaryocytic differentiation, but presence of PMF history and common genetic abnormality, *i.e.*, *JAK2* V617F mutation, suggest that extramedullary blastic transformation, which is occasionally seen in CML, can also occur in PMF. Therefore, we conclude that understanding the variations in the pathogeneses of PMF and MyS is important.

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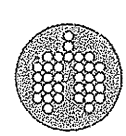

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Reduced larger von Willebrand factor multimers at dawn in OSA plasmas reflect severity of apnoeic episodes

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ABSTRACT: Plasma von Willebrand factor (VWF), produced in and released from vascular endothelial cells by various stimuli including hypoxia, induces platelet aggregation under high shear stress and plays dual pivotal roles in haemostasis and thrombosis within arterioles, which are regulated by the size of vWF multimers (VWFMs).

Patients with obstructive sleep apnoea (OSA) have increased risk of thrombotic cardiovascular events, but the pathogenesis is unclear. We examined the relationship between VWF and OSA by measuring VWF antigen (VWF:Ag), VWFMs, VWF collagen binding activity (VWF:CB) and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13. A total of 58 OSA patients were enrolled. Blood samples were collected before sleep, after sleep, and after one night of nasal continuous positive airway pressure therapy.

Based on VWFM analysis, OSA patients were classified into three groups; consistently normal VWFMs (group 1, n=29), increased high molecular weight (HMW)-VWFMs at 06:00 h (group 2, n=18), and decreased or absent HMW-VWFMs at 06:00 h (group 3, n=11). Patients in group 3 had significantly worse apnoea/hypopnoea index; VWF:CB followed a similar pattern. We observed a significant decrease in platelet count between 21:00 h and 06:00 h in OSA patients, potentially associated with reduced larger VWFMs together with decreased VWF:Ag levels. Severe OSA may contribute to an arterial pro-thrombotic state.

KEYWORDS: ADAMTS13, obstructive sleep apnoea, von Willebrand factor

Obstructive sleep apnoea (OSA) is characterised by the collapse of the upper airway and associated intermittent hypoxia during sleep [1]. OSA is associated with excessive daytime sleepiness and cardiovascular disease. Patients with OSA often suffer from obesity, hypertension, hyperlipidaemia, and impaired glucose tolerance, and OSA is an independent risk factor for cardiovascular diseases [2–4]. Consistent with this, cardiovascular risk returned to baseline in OSA patients treated with nasal continuous positive airway pressure (CPAP), whereas those with severe untreated OSA maintained a high risk [5]. Recently, some association of OSA with venous thromboembolism in regard to pulmonary embolism has been implicated [6, 7]. However, the mechanism of OSA-associated thrombosis might be multifactorial, and in fact has not been evaluated on a basis of arterial thrombosis, which is generated under high shear stress in microvasculatures, where von Willebrand factor (VWF) plays a critical role as a molecular glue that facilitates platelet aggregation or thrombi.

VWF is a macromolecular plasma protein, which is exclusively produced in and released from vascular endothelial cells, and exerts pivotal effects on both haemostasis and thrombosis. VWF assembles into unusually large VWF multimers (UL-VWFMs) consisting of identical 250 kDa subunits, before its release into the circulation. Under normal circumstances, UL-VWFMs are rapidly cleaved by a specific plasma protease, ADAMTS13 (a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13), under the high shear stress generated in the microvasculature; consequently, VWF circulates in the plasma as a heterogeneous family of multimers ranging in size from 500 to 15,000 kDa. UL-VWFMs play an essential role in primary haemostasis by binding platelets to denuded vascular endothelial tissue. However, in the absence of ADAMTS13 activity (ADAMTS13:AC) due to gene mutation or acquired autoantibodies, UL-VWFMs remain uncleaved and generate platelet hyperaggregation. Uncleaved UL-VWFMs lead to the formation of vast platelet thrombi, known as thrombotic

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TABLE 1 Characteristics of patients with obstructive sleep apnoea (OSA) and sleep controls

	OSA	Sleep controls	p-value
Sex n (M/F)	58 (55/3)	25 (22/3)	NS
Blood type			NS
A	18	12	
B	8	2	
O	26	8	
AB	6	3	
Age yrs	44.7±9.9	38.3±7.1	<0.01
BMI kg·m^{-2}	28.2±3.7	27.7±3.0	NS
AHI	50.5±22.2	4.5±2.8	<0.01
ODI3%	41.6±19.9	7.8±5.1	<0.01
Lowest Sp,O_2 %	76.0±10.0	88.8±5.0	<0.01
Systolic blood pressure mmHg	129±16	122±28	NS
Diastolic blood pressure mmHg	82±12	81±10	NS
vWF:Ag levels % at 06:00 h	103.1±61.4	143.5±63.8	<0.01
ADAMTS13:AC levels % at 06:00 h	56.8±22.6	61.7±20.6	NS

Data are presented as mean±SD, unless otherwise stated. M: males; F: females; BMI: body mass index; AHI: apnoea/hypopnoea index; ODI3%: oxygen desaturation index ⩾3%; Sp,O_2: arterial oxygen saturation measured by pulse oximetry; vWF:Ag: von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospondin type 1 motifs 13 activity; NS: not significant.

thrombocytopenic purpura, a life-threatening generalised disease [8–11].

It is now well established that high plasma levels of VWF antigen (VWF:Ag) are linked with an increased risk for ischaemic heart disease and ischaemic stroke [12–14]. Furthermore, the relative risks of stroke and acute myocardial infarction are higher in individuals with lower ADAMTS13:AC [14, 15]. Furthermore, hypoxia leads to increased VWF release from cultured vascular endothelial cells, both directly, by up regulating VWF expression, and indirectly *via* autocrine and paracrine signalling downstream of hypoxia-induced inflammatory cytokines including interleukin (IL)-6, IL-8, and tumour necrosis factor-α [16, 17]. Despite these important reports of hypoxia-induced VWF secretion, no subsequent studies have addressed the relationship between VWF and the severity of OSA [18, 19]. In particular, no studies have been performed on plasma samples obtained in chronological order relevant to the sleep cycle.

In this study, we sequentially analysed plasma VWF:Ag levels, VWFM patterns, and ADAMTS13:AC in OSA patients not only before and after sleep, but also before and after CPAP treatment. We found that the reduced larger VWFMs together with decreased VWF:Ag levels in the plasma of OSA patients taken at dawn correlate with the clinical severity of apnoeic episodes.

PATIENTS, MATERIALS AND METHODS

Patients

Between February 2004 and April 2011, 284 patients received full standard diagnostic polysomnography (PSG) at Nara Medical University Hospital (Nara, Japan). Among them, 86 patients were diagnosed with normal or mild OSA (apnoea/

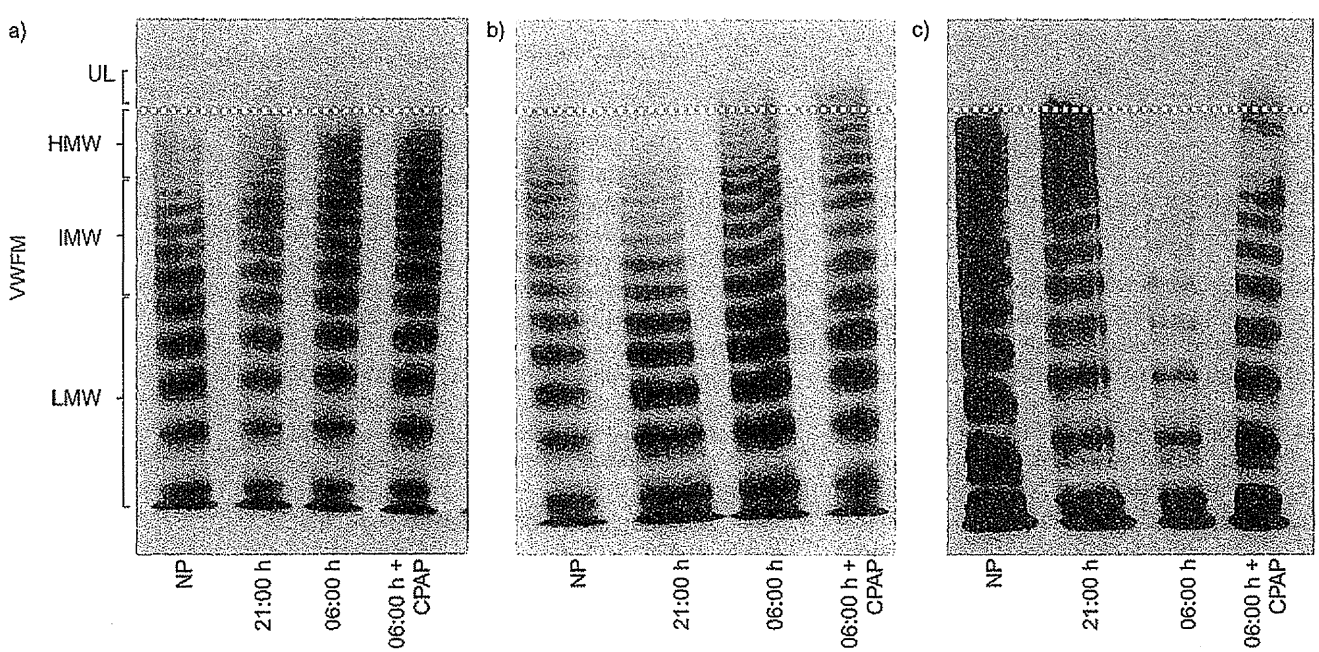

FIGURE 1. Patterns of von Willebrand factor multimers (VWFMs) corresponding to three patient groups. Obstructive sleep apnoea (OSA) patients were categorised into three groups based on the results of VWFM analysis, using sequential samples. Representative results from each group are shown. a) Group 1, patients (n=29) showed a consistently normal pattern of VWFMs. b) Group 2, patients (n=18) had increased, unusually large (UL)- and high molecular weight (HMW)-VWFMs at 06:00 h compared to 21:00 h. c) Group 3,patients (n=11) had decreased UL- and HMW-VWFM at 06:00 h compared to 21:00 h.

hypopnoea index (AHI) <15), and 198 patients were diagnosed with moderate or severe OSA (AHI ⩾15) and received nasal CPAP therapy. Within the latter group, 140 patients with the following underlying diseases were excluded: stroke, coronary artery disease, asthma, chronic obstructive pulmonary disease, arthritis, autoimmune disease, rhinitis, and malignant diseases. The 58 remaining OSA patients were enrolled in this study; detailed clinical information for these 58 patients is shown in table S1. Written informed consent was obtained from all patients, and the study was approved by the Human Subjects Ethics Committee of Nara Medical University (No. 04-012). 25 healthy volunteers (88% male), as shown in table 1, that had undergone PSG studies without OSA were also enrolled and used as the sleep controls.

Blood sampling

Plasma samples were collected from OSA patients at three time points throughout the day; 21:00 h before PSG, at 06:00 h after the PSG without CPAP, and at 06:00 h after CPAP treatment. For the sleep control subjects, plasma samples were collected at 06:00 h. Blood was collected in plastic tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing a tenth volume of 3.8% trisodium citrate an anticoagulant, and platelet-poor plasma was prepared by centrifugation at 3,000 × g for 15 min at 4°C. Aliquots were stored at -80°C prior to use. To obtain platelet counts, blood was collected into plastic whole blood tubes with spray-coated EDTA (Becton, Dickinson and Co.) tubes containing EDTA as an anticoagulant and analysed with a Coulter counter (Beckman Coulter, Tokyo, Japan).

Sleep study

PSG was performed using a computerised polysomnography system (Alice 4; Respironics, Pittsburgh, PA, USA). Data acquisition began at 21:00 h and continued until 06:00 h the following day. Apnoea was defined as a cessation of airflow for ⩾10 s, and hypopnoea was defined as a decrease in airflow at least 50% for a minimum of 10 s or a clear decrease in airflow (⩾20%) followed by either oxygen desaturation ⩾3% or signs of physiological arousal. The AHI was calculated as the number of apnoea/hypopnoea events per hour of total sleeping time. We also calculated the oxygen desaturation index ⩾3% (ODI3%), defined as the number of ⩾3% dips in oxygen saturation per hour of sleep.

During the night, following diagnostic PSG, patients were treated with nasal CPAP (REMstar Auto; Respironics), with PSG monitoring. Apnoeic episodes were substantially reduced or eliminated during treatment with nasal CPAP.

Analyses of VWF:Ag, VWF, and VWF:CB

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, Glostrup, Denmark) [20]. The VWF:Ag level contained in 1 mL of pooled normal human plasma was defined as 100%; VWF:Ag levels in the 20 healthy controls were 102±33% (mean±SD) [21].

VWFMs were analysed by sodium dodecyl sulphate-1.2% agarose gel electrophoresis followed by Western blotting with luminographic detection [22, 23]. The blots were scanned and subjected to densitometric analysis using ImageJ (National Institutes of Health (NIH), Bethesda, MD, USA). Multimers were classified as low molecular weight (LMW-VWFMs; corresponding to bands 1-5 in VWFM analysis), intermediate molecular weight (IMW-VWFMs; bands 6-10), and high molecular weight (HMW-VWFMs; bands ⩾11) [24]. High molecular weight bands that were not detected in normal plasma (NP) were defined as UL-VWFMs. The levels of LMW-, IMW- and HMW-VWFMs were calculated using NIH ImageJ. For quantitative analyses, we calculated the ratios of the densities of VWFMs, LMW, IMW, and HMW relative to total VWFM density. Further, multimeric VWF:Ag levels were calculated by multiplying VWF:Ag level by the LMW, IMW, and HMW ratios.

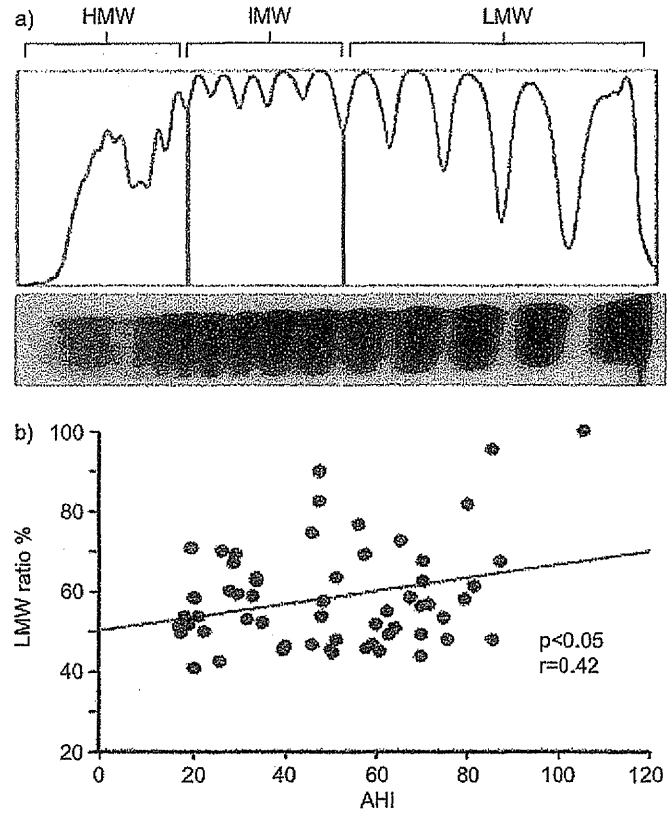

FIGURE 2. Relationship between low molecular weight (LMW) von Willebrand factor multimers (VWFMs) to total VWFMs (LMW ratio) and hypoxia. a) Quantitative analysis of VWFMs was performed by calculating the density of LMW-VWFMs relative to total M density. A representative result of VWF analysis at 06:00 h is shown. b) The LMW ratio of obstructive sleep apnoea patients was significantly correlated to apnoea/hypopnea index (AHI). IMW: intermediate molecular weight.

The plasma VWF collagen binding activity (VWF:CB) was measured using an enzyme immunoassay using a commercially available kit (VWF-CBA ELISA, PROGEN Biotechnik GmbH, Heidelberg, Germany) according to the manufacturer's instructions.

Assay of ADAMTS13:AC

ADAMTS13:AC was determined using a commercially available chromogenic ELISA/ACT (Kainos Co., Tokyo, Japan). The detection limit of this assay was 0.5%; the values obtained from 55 healthy controls were 99.1±21.5% (mean±SD) [25].

Statistical analysis

Laboratory data are expressed as the mean±SD. Comparisons between OSA patients and controls were analysed using the

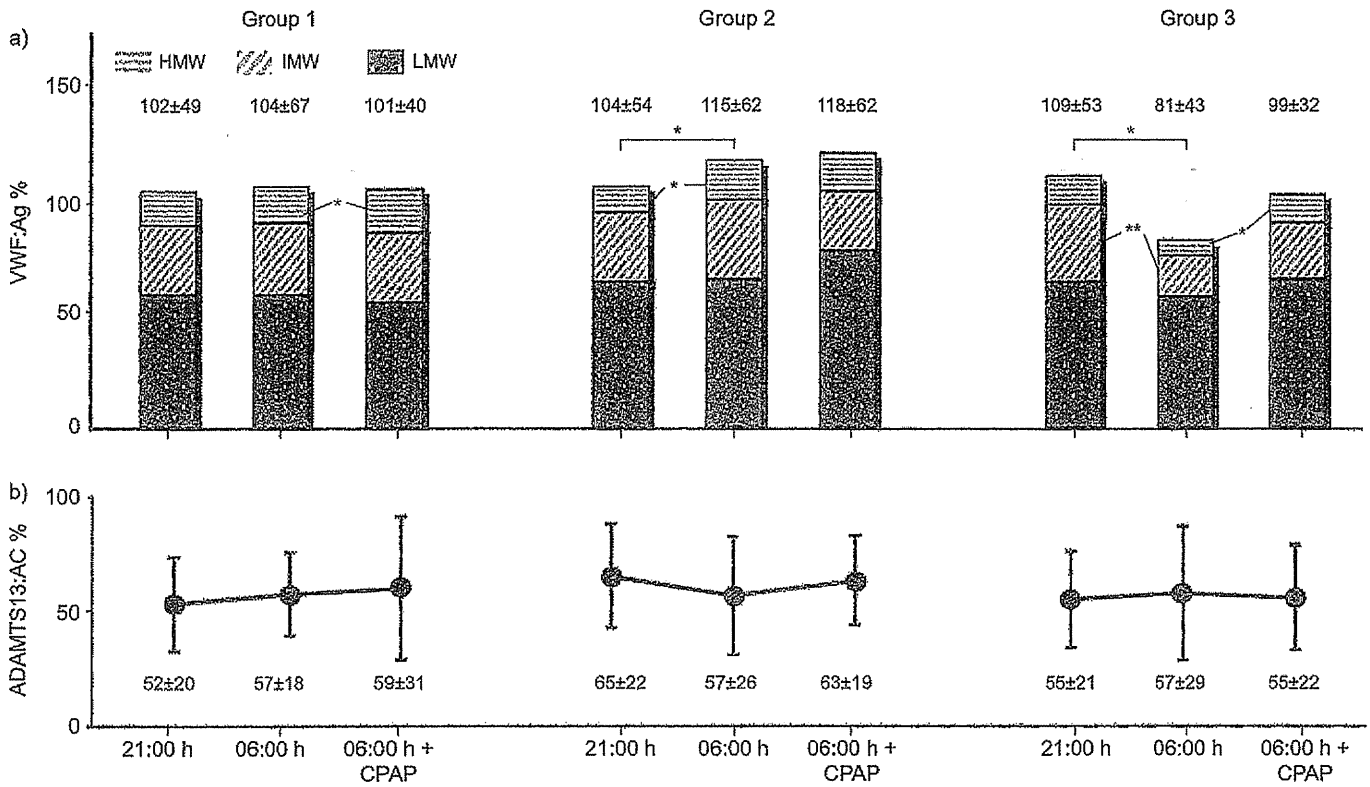

FIGURE 3. Changes in serial von Willebrand factor antigen (VWF:Ag) levels and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity (ADAMTS13:AC) in groups 1–3. VWF:Ag levels were divided into high molecular weight (HMW)-, intermediate molecular weight (IMW)-, and low molecular weight (LMW)-VWFM groups by multiplying the VWF:Ag level by the results of the multimeric analyses. Data are presented as mean±SD . Groups were first compared using the Kruskal-Wallis H-test; significantly different groups were then analysed using the Mann-Whitney U-test. *: $p<0.05$; **: $p<0.01$.

Mann-Whitney U-test or Chi-square test. All comparisons among the three groups were tested for statistical significance using the Kruskal-Wallis H-test or Chi-square test, with Yates' correction for 2×3 tables; significant differences between the three groups (overall $p<0.05$) were further analysed using the Mann-Whitney U-test or Chi-square test. All analyses were carried out using StatView (SAS Institute Inc., Cary, NC, USA). A p-value <0.05 was considered significant.

RESULTS

Characteristics of patients with OSA and controls

The demographics and sleep characteristics of patients with OSA and controls are shown in table 1. Patients with OSA were slightly older than the control population but were otherwise similar demographically. 18, seven, and four patients in the OSA group were being treated for hypertension, hyperlipidaemia, and diabetes mellitus, respectively, but no diabetic patients were receiving insulin therapy. Based on the PSG results, the two populations differed significantly with respect to AHI, ODI3%, and lowest Sp,O_2 %.

Plasma VWF:Ag levels at 06:00 h were significantly lower in patients with OSA compared with the controls, but plasma ADAMTS13:AC at 06:00 h did not differ between these groups. Interestingly, the plasma ADAMTS13:AC at 06:00 h in both OSA patients and sleep controls were lower than those of the above mentioned healthy controls ($p<0.01$).

Chronological changes of plasma VWFM patterns categorise the patients with OSA into three groups

We analysed VWFM patterns in plasmas taken from OSA patients, obtained at 21:00 h and 06:00 h following sleep with or without CPAP. Based on these results, we categorised the patients with OSA into three groups (fig. 1). Patients in group 1 (n=29) had a consistently normal pattern of VWFM, almost indistinguishable from that of the sleep controls (n=6). Patients in group 2 (n=18) exhibited reduced HMW-VWFMs at 21:00 h and persistent UL-VWFMs at 06:00 h, with or without CPAP. Patients in group 3 (n=11) had normal VWFM patterns at 21:00 h, reduced predominantly HMW-VWFMs at 06:00 h without CPAP, and returned to a normal VWFM pattern after CPAP therapy.

The decrease in HMW-VWFMs and concomitant increase in LMW-VWFMs could reflect either enhanced proteolysis by ADAMTS13 or extensive consumption secondary to platelet aggregation. Therefore, we first calculated the ratio of LMW-VWFMs to total VWFMs (LMW ratio) at 06:00 h without CPAP (fig. 2), and subsequently determined the relationship between LMW ratio and AHI. As shown in figure 2, these two parameters are significantly correlated ($p<0.05$), suggesting that the

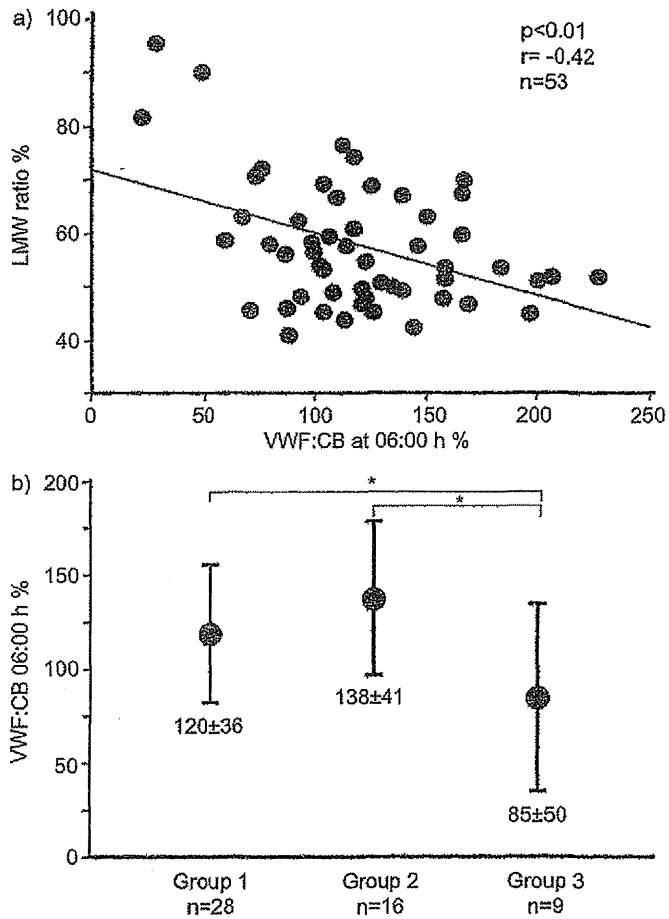

FIGURE 4. Relationship von Willebrand factor (VWF) collagen binding activity (VWF:CB) and ratio of low molecular weight (LMW)-VWF multimers (Ms) to total VWFMs (LMW ratio) and comparison of vWF:CB at 06:00 h in each group. vWF:CB was measured in 53 out of 58 obstructive sleep apnoea (OSA) patients. a. Significant inverse correlation between LMW ratio and VWF:CB at 06:00 h in OSA patients. b) VWF:CB at 06:00 h in group 3 was significantly lower than in groups 1 and 2. Data are presented as mean±SD *: $p<0.05$.

degree of hypoxia during apnoeic events is related to vFWMs processing and/or consumption.

Chronological changes of plasma levels of VWF:Ag, VWFM ratio, and ADAMTS13:AC in three patient groups with OSA

Plasma levels of VWF:Ag at 21:00 h, 06:00 h without CPAP, and 06:00 h with CPAP were determined in all three groups of OSA patients. As shown in figure 3, plasma VWF:Ag levels were almost unchanged in group 1 patients, but significantly increased between 21:00 h and 06:00 h in group 2 patients. Notably, VWF:Ag levels remarkably decreased between 21:00 h and 06:00 h in group 3.

We then determined levels of HMW, IMW, and LMW in all three groups. In group 1, HMW-VWFM showed a slight increase at 06:00 h with CPAP, relative to 06:00 h without CPAP. In group 2, HMW-VWFMs significantly increased at 06:00 h compared to 21:00 h confirming the results of the VWFM analysis used for defining groups 1–3. Consistent with this, in group 3, the IMW-VWFMs at 06:00 h was significantly lower than that at 21:00 h; CPAP treatment reversibly increased the HMW-VWFM at 06:00 h, in accordance with the increase in plasma VWF:Ag level.

In contrast, no change in the plasma ADAMTS13:AC was seen at 21:00 h, 06:00 h, or 06:00 h with CPAP in any of the three groups. These data argue that consumption of the HMW-VWFMs occurred overnight in OSA patients.

Plasma levels of VWF:CB activity

We observed dynamic chronological changes in plasma VWF:Ag levels and VWFM patterns in our subjects, especially in group 3. VWF:CB represents a biological function of VWF, in which HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. In this study, we were able to examine plasma VWF:CB levels in 53 out of 58 OSA patients. As expected, plasma levels of VWF:CB at 06:00 h without CPAP were inversely correlated with the LMW ratio ($p<0.01$), as shown in figure 4. Furthermore, as shown in figure 4, plasma levels of VWF:CB at 06:00 h was significantly lower in group 3 (85±50%) than in either group 1 (120±36%) or group 2 (138±41%). These results argue that structurally and functionally impaired VWFMs were present at 06:00 h in group 3 patients.

Decreased platelet counts at dawn in the untreated patients with OSA

A pair of platelet counts at 21:00 h and 06:00 h without CPAP was determined in 31 of the 58 OSA patients and in six of the 25 sleep controls, all of whom were involved in the later phase of this study. To correct for a possible hydration effect during sleep, we calculated the ratio of platelet count to haematocrit. The ratios in sleep controls did not exhibit significant changes between 21:00 h and 06:00 h (fig. 5), whereas they were lower at 06:00 h in untreated OSA patients ($p<0.01$) (fig. 5). However, none of the patients who received CPAP treatment developed overt clinical signs of thrombotic complications. These results suggest that platelet consumption, to a lesser extent, might occur during sleep without distinct thrombotic symptoms in untreated OSA patients.

Patient characteristics of groups 1, 2, and 3

Table 2 summarises the demographic and measured parameters of OSA patients categorised into groups 1–3. These three groups did not differ demographically, but AHI was significantly higher in group 3 than in groups 1 and 2. ODI3% in group 3 was also significantly higher than in group 1. These results unambiguously indicate that patients in group 3, who exhibit lower levels of large VWFM at 06:00 h represent the highest severity of OSA among the three groups.

Consistent with these results, decreased plasma levels of VWF:Ag in the two different time intervals (06:00 h and 21:00 h) was remarkable in group 3, in comparison to those in groups 1 and 2. Interestingly, the differences of LMW ratio in the two times (06:00 h and 21:00 h) was significantly higher in group 3 than those of groups 1 or 2. These results indicated that decreased VWF:Ag at 06:00 h was caused primarily by the reduction in larger VWFMs. Alternatively, no significant change in ADAMTS13:AC between the two times (06:00 h and 21:00 h) was observed in group 3, whereas such a change was observed in groups 1 and 2, leaving the physiological relevance unaddressed.

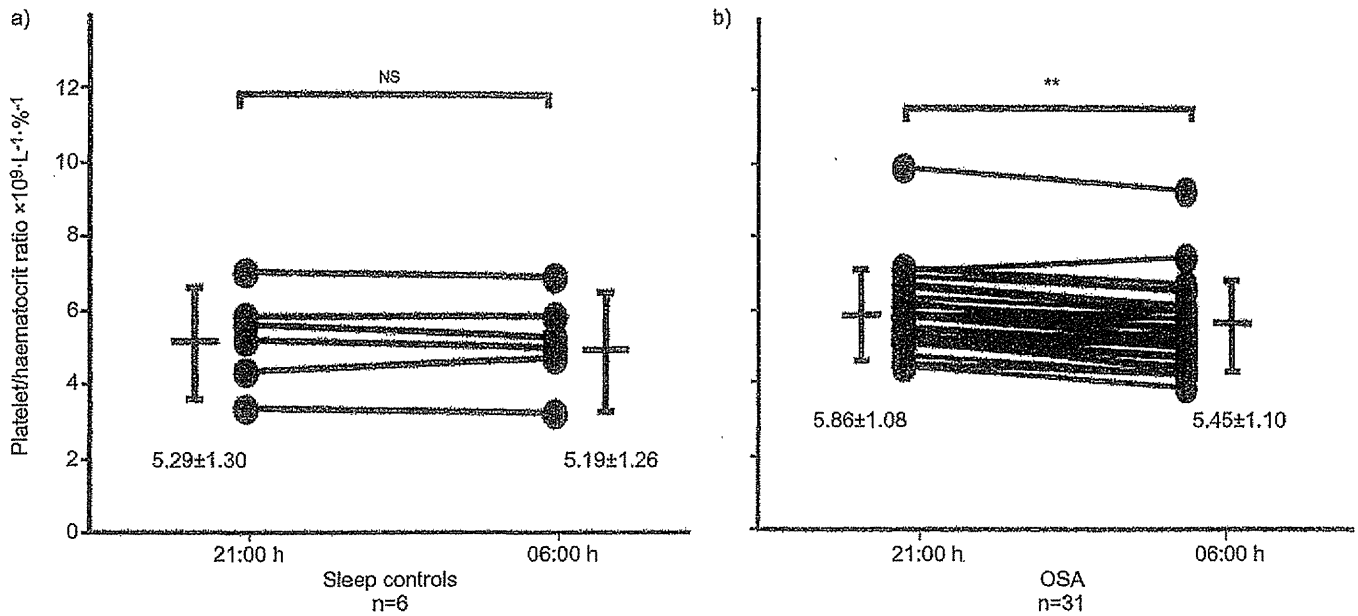

FIGURE 5. Overnight platelet counts to haematocrit ratios decreased in patients with obstructive sleep apnoea (OSA). Platelet counts were normalised to the patient's haematocrit to control for differences in hydration status. Ratios of platelet count to haematocrit were obtained at 21:00 h and 06:00 h in a) six sleep controls and in b) 31 OSA patients, both without CPAP treatment. In the sleep controls, the ratios did not change between time points. In the OSA patients, the ratio exhibited significant changes between time points. Data are presented as mean±SD. NS: nonsignificant. **: $p<0.01$.

Relationship of AHI and groups 1–3 of VWFM patterns in OSA patients

AHI is an excellent means of showing OSA severity, here we have used it to categorise three groups: moderate ($15 \leqslant AHI<30$), severe ($30 \leqslant AHI<60$), and extremely severe (AH $I \geqslant 60$). As shown in table 3, OSA patients with group 1 and 2 consisted of those with variable AHI levels. Notably, none of the OSA patients within group 3 had an AHI 15~<30, and they uniformly

TABLE 2 Characteristics and different parameter between 21:00 h and 06:00 h of patients with obstructive sleep apnoea (OSA) in groups 1–3

	Group			Overall p-value
	1	2	3	
Sex M/F	28/1	18/0	9/2	NS
Blood type				NS
A	7	4	7	
B	4	4	0	
O	14	7	4	
AB	4	3	0	
Age yrs	46.0±9.6	42.9±9.7	44.2±11.3	NS
AHI	43.1±20.0	51.4±19.6	68.7±22.6	<0.05*
ODI3%	35.7±16.2	44.1±19.3	53.2±21.5	<0.01#
Differences in time intervals 06:00 and 21:00 h				
VWF:Ag %	2.1±34.8	10.8±22.0	-28.1±40.6	<0.05¶
LMW ratio %	-0.27±5.24	-4.46±8.69	16.69±16.92	<0.01¶
ADAMTS13:AC %	4.4±13.1	-8.5±25.9	2.4±21.4	<0.05+
Plt/Ht $\times 10^9 \cdot L^{-1} \cdot \%^{-1}$	-0.045±0.036 (n=15)	-0.034±0.038 (n=10)	-0.043±0.029 (n=6)	NS

Data are presented as n or mean±SD, unless otherwise stated. M: males; F: females; AHI : apnoea/hypopnea index, ODI3%: oxygen desaturation index ⩾3%; vWF:Ag : von Willebrand factor antigen; LMW ratio: the ratio of low molecular weight-VWFMs to total VWFMs; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospondin type 1 motifs 13 activity; Plt/Ht: platelet count to haematocrit ratio. NS: not significant. *: $p<0.05$ between groups 1, 2 and 3; #: $p<0.01$ between groups 1 and 3; ¶: $p<0.01$ between groups 1, 2 and 3; +: $p<0.05$ between group 1 and 2.

TABLE 3 Characteristics and thrombotic parameters of patients classified with apnoea/hypopnoea index (AHI)

	15≤AHI<30	30≤AHI<60	AHI≥60	Overall p-value
Patients n	15	22	21	
Sex M/F	15/0	21/1	19/2	NS
Age yr	43.7±12.0	42.9±9.7	44.2±11.3	NS
ODI3%	19.2±4.9	36.2±10.9	63.3±9.4	<0.01**
VWFM group				
1	12 (80)	8 (36)	9 (43)	<0.05*
2	3 (20)	10 (45)	5 (24)	NS
3	0	4 (18)	7 (33)	<0.05#
VWF:Ag at 06:00 h %	98.5±49.1	98.5±55.7	111.3±75.5	NS
ADAMTS13:AC at 06:00 h %	58.1±20.2	55.2±21.9	57.6±25.6	NS
VWF:CB at 06:00 h U·mL^{-1}	1.29±0.39 (n=13)	1.23±0.50 (n=19)	1.09±0.38 (n=19)	NS
Plt/Ht at 06:00 h ×10^{9}·L^{-1}·%$^{-1}$	0.526±0.093 (n=10)	0.549±0.138 (n=13)	0.561±0.087 (n=8)	NS

Data are presented as mean±SD or n (%), unless otherwise stated. M: males; F: females; ODI3%: oxygen desaturation index ≥3%; VWFM: von Willebrand factor multimer; VWF:Ag : von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; Plt/Ht: platelet count to haematocrit ratio; NS: not significant. *: p<0.05 between 15≤AHI<30 and 30≤AHI<60, AHI≥60, **: p<0.01 between all AHI groups; #: p<0.05 between 15≤AHI<30 and AHI≥60.

had AHI ≥30 and more predominantly with AHI ≥60. The incident for group 1 patients was lower in AHI groups of 30≤AHI<60 and AHI≥60 than those of 15≤AHI<30 (p<0.05). In contrast, the incident for group 3 was higher in AHI≥60 than those of 15≤AHI<30 (p <0.05). No significant relationship between AHI score and each parameter such as VWF, ADAMTS13, or platelet count was found.

DISCUSSION

Plasma VWF:Ag levels increase after the age of 40 yrs in normal individuals; by the age of 60 yrs they can have reached ~120–140% of the healthy normal baseline [26]. The mean age of OSA patients enrolled in this study was 44.7 yrs, whereas that of control subjects was 38.3 yrs. However, the plasma VWF:Ag levels collected at 06:00 h were significantly lower for OSA patients than for control subjects (table 1). In contrast, plasma ADAMTS13 activity decreases after the age of 40 yrs in normal individuals [27]. Among our study patients and controls, plasma ADAMTS13:AC was lower than in healthy controls aged between 20-40 yrs (p<0.01), indicating that these two groups did not significantly differ (table 1).

Given the observed differences in VWF:Ag levels between OSA patients and control subjects, we analysed VWFM patterns chronologically at three time points: at 21:00 h and at 06:00 h either with or without overnight CPAP treatment. As expected, a majority of OSA patients (29 (50%) out of 58) had consistently normal VWFM patterns, categorised as group 1. Two smaller groups of patients had increased UL- and HMW-VWFM (18 (31%) out of 58) or decreased UL- and HMW-VWFM (11 (19%) out of 58) at 06:00 h; these were categorised as group 2 or group 3, respectively. The ratio of LMW-VWFM to total VWFM, termed the LMW ratio, is a determination of the relative amount of degraded VWFM; in our study population, the LMW ratio correlated significantly with the AHI.

The increased LMW ratio seen in OSA patients could arise from reduced production of VWF by vascular endothelial cells, increased clearance of HMW-VWFM from the circulation, or consumption during thrombosis. However, *in vitro* studies have clearly shown that VWF expression by cultured vascular endothelial cells is increased under conditions of hypoxia; it is unlikely that patients with OSA, a condition of intermittent hypoxia, would exhibit decreased expression of VWF overnight [17]. Additionally, no differences were seen in the plasma ADAMTS13:AC in any group at any time-point, suggesting that enhanced proteolysis of HMW-VWFM was not occurring. Therefore, we hypothesised that the elevated LMW ratio seen in our OSA patients was likely to be due to an enhanced degradation or consumption of HMW-VWFM.

The cause of thrombotic complications in OSA patients might be multifactorial, but in this study we have clearly indicated that VWF appears to play an essential role in the thrombogenesis in a certain population categorised as group 3. Although the mechanism is not yet fully elucidated, the high VWFMs released upon hypoxia from vascular endothelial cells is a most plausible factor. Thus, severe OSA could be a risk factor for both arterial and venous thrombosis as described in the introduction.

To better understand whether some degree of thrombosis was occurring overnight in untreated OSA patients, we determined platelet counts in 31 out of 58 patients; we observed a significant decrease in platelet count between 21:00 h and 06:00 h. This decrease was associated with reductions in both the plasma VWF:Ag levels and HMW-VWFM in group 3. Quantitative analyses of VWFMs in group 3 showed that levels of HMW-VWFMs increased significantly after CPAP treatment, compared with measurements taken at 06:00 h without CPAP. This is consistent with low-level consumption of UL- and HMW-VWFMs by microvascular thrombus formation and/or platelet aggregation during sleep in OSA patients; CPAP therapy might reduce such consumption. However, no patients have developed overt clinical signs of thromboembolic complications; therefore, we prefer to use the term "pre-clinical platelet consumption"

to describe this phenomenon. This may represent a baseline pro-thrombotic state in OSA patients that can be corrected by CPAP therapy.

In this study, the chronological analyses have unanimously indicated that reduced large VWFMs in plasmas at dawn reflect the clinical severity of apnoea in OSA patients. The results obtained by VWFM analysis were solid, but the procedure was time consuming and requires a high technical skill to perform. A reliable high-throughput method would be necessary for routine clinical use. In this regard, the assay for VWF:CB is a promising candidate for such a method, because HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. Our results indicated that VWF:CB at 06:00 h correlated well with VWFM patterns, and was consistent with earlier assignment of subjects to groups 1–3. Thus, through this study we have provided the first convincing evidence that VWF at dawn in group 3 was impaired not only structurally but also functionally, presumably due to hypoxia-induced release and consumption of VWF. This process might also involve platelet aggregation and consumption, even though the patients were asymptomatic. Thus, large scale studies, together with chronological measurements of platelet counts and VWF:CB, would be the focus in the following studies.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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PLoS one

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

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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 information; 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

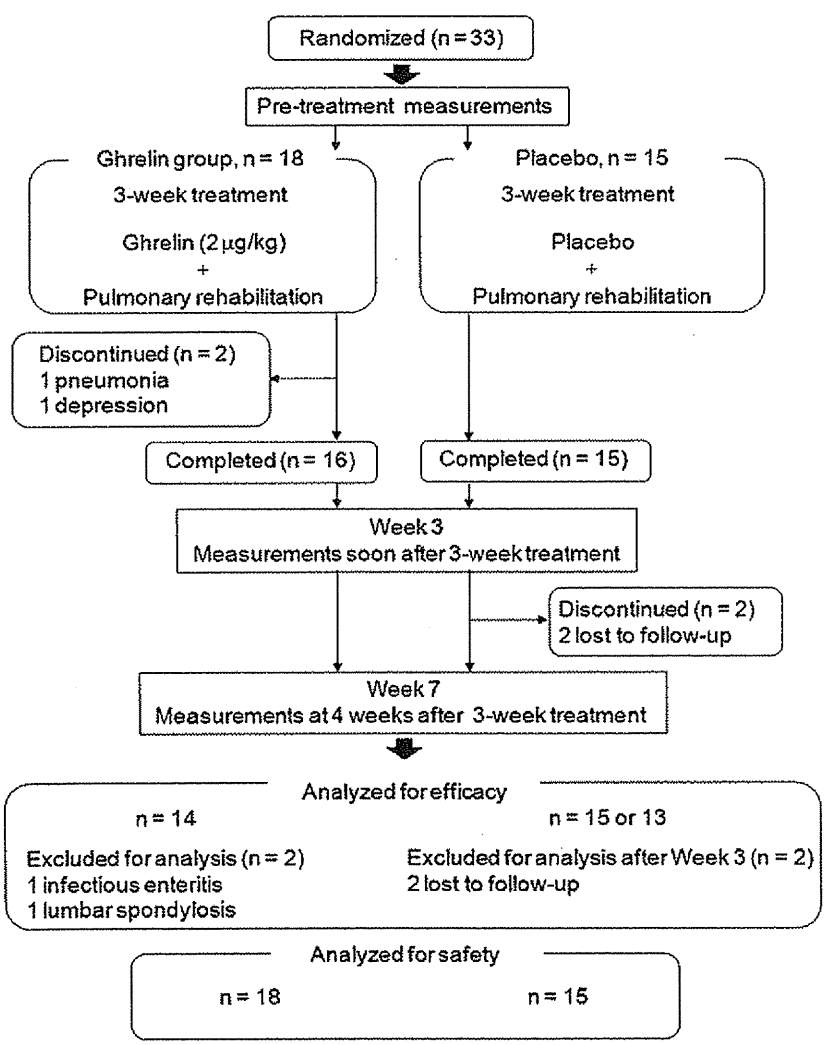

Figure 1. Trial profile.
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informed consent (in Japanese). The inclusion criteria were as follows: 1) severe to very severe COPD (forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) of less than 70% and FEV1 percent predicted of less than 50%); 2) underweight (body mass index (BMI)<21 kg/m^2); 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 ≥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 μg/kg) or placebo twice a day for 3 weeks in hospital. The administration of ghrelin (2 μg/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 μg 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-MWD, 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; FEV_1 = 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.

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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].

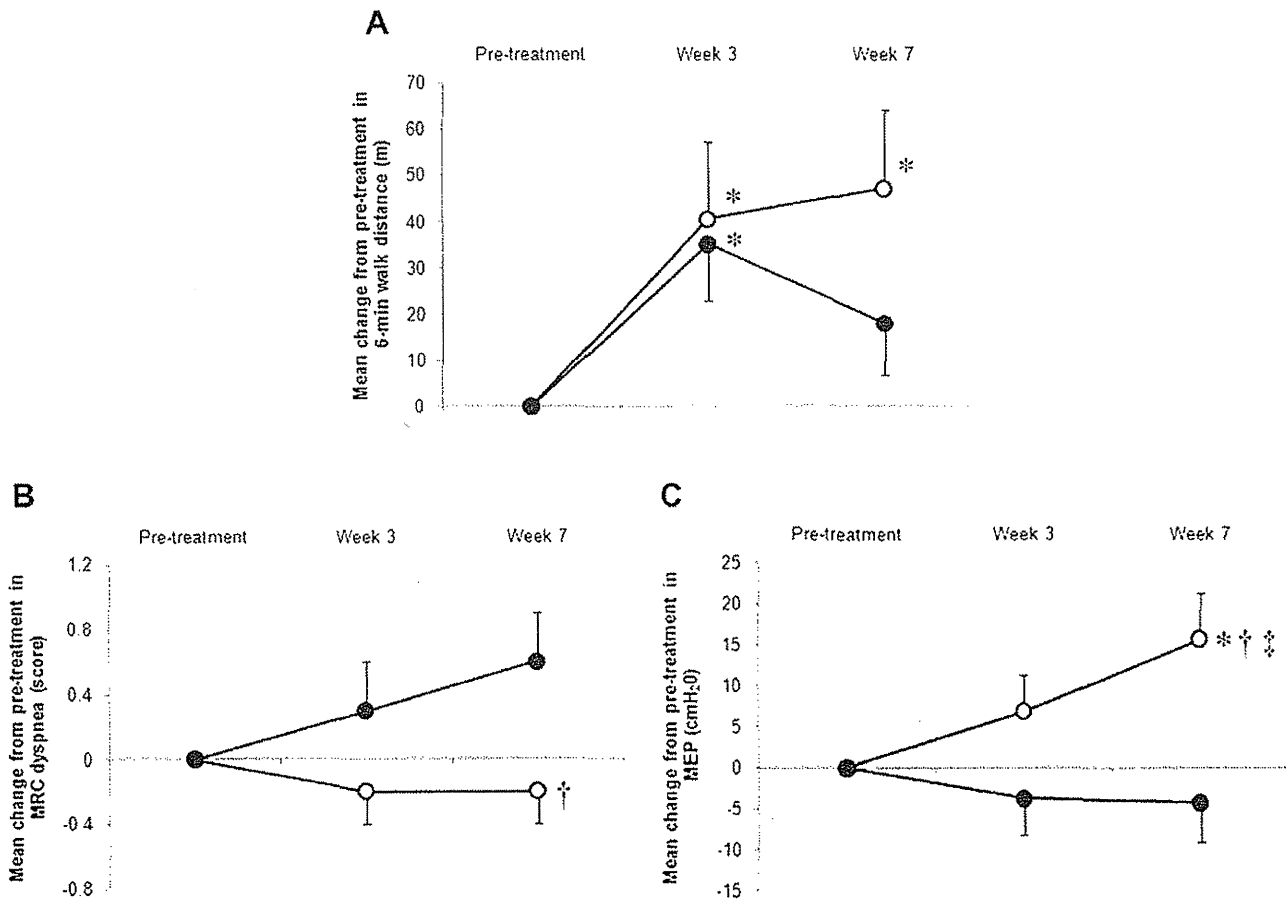

Figure 2. Change from pre-treatment in 6-min walk distance (6-MWD), Medical Research Council (MRC) score, and maximal expiratory pressure (MEP) over time. Open circles, ghrelin; closed circles, placebo. Data are presented as mean differences±SE. * $p<0.05$: change between pre- and post-treatment (within-group 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 ($F(2, 51) = 4.17$, $p = 0.021$).
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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_20	−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_20	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; FEV_1 = 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.

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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±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

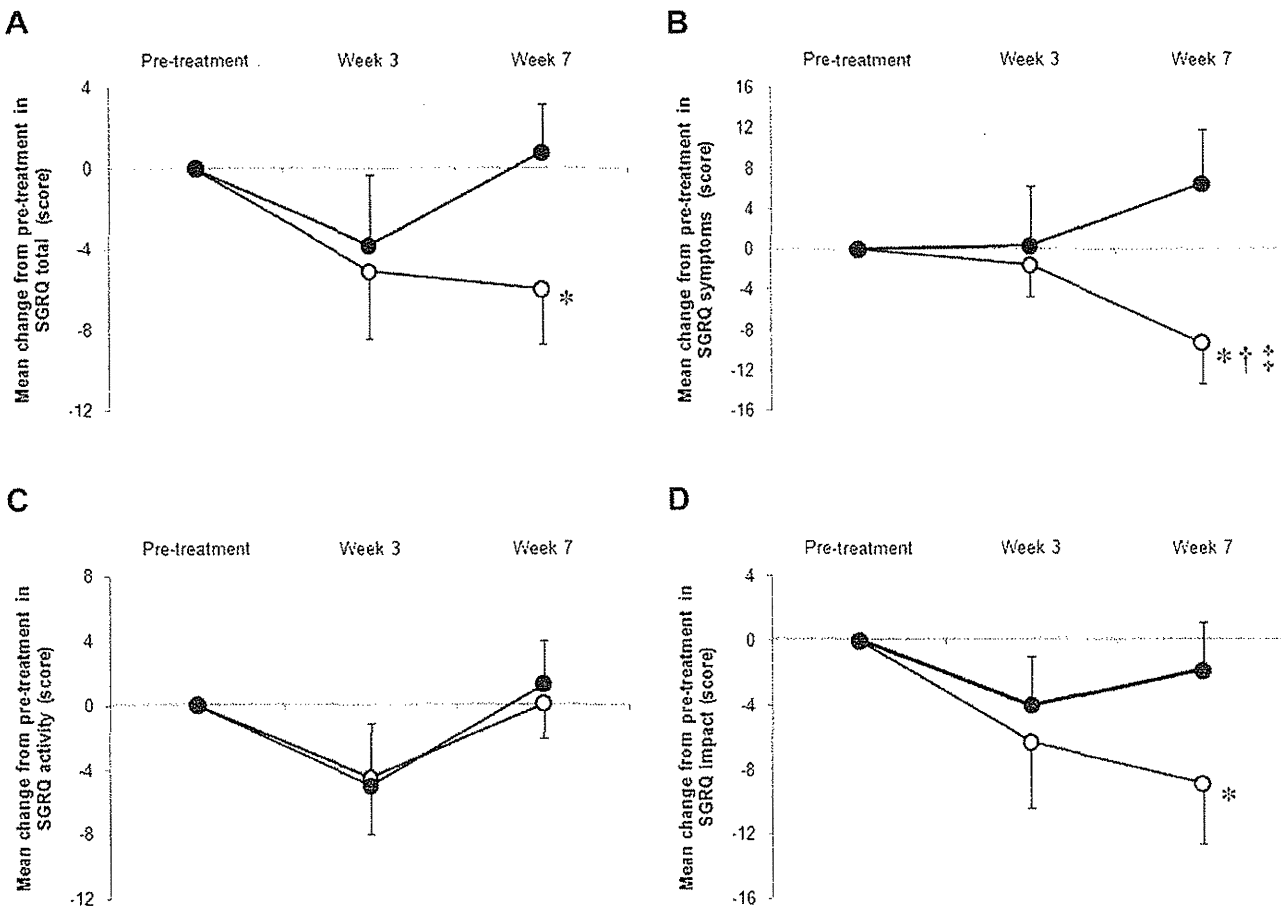

Figure 3. Change from pre-treatment in St. George Respiratory Questionnaire (SGRQ) scores over time. Open circles, ghrelin; closed circles, placebo. Data are presented as mean differences±SE. * $p<0.05$: change between pre- and post-treatment (within-group difference). † $p<0.05$: change between pre-treatment 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 ($F(2, 51) = 3.19$, $p = 0.049$).
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(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±SD, 18.3±2.1 kg/m^2). 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±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±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±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