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Original Research

Reduced larger VWF multimers at dawn in OSA plasmas reflect severity of apneic 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 hemostasis and thrombosis within arterioles, that are regulated by the size of VWF multimers (Ms).
Patients with obstructive sleep apnea (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, VWFMs, VWF collagen binding activity (VWF:CB), and ADAMTS13. Fifty-eight OSA patients were enrolled. Blood samples were collected before sleep, after sleep, and after one night of nasal continuous positive airway pressure (CPAP) therapy.
Based on VWFM analysis, OSA patients were classified into 3 groups; consistently normal VWFM (Group 1, n=29), increased high molecular weight (HMW)-VWFM at 6 am (Group 2, n=18), and decreased or absent HMW-VWFM at 6 am (Group 3, n=11). Patients in Group 3 had significantly worse apnea-hypopnea index; VWF:CB followed a similar pattern. We observed a significant decrease in platelet count between 9 pm and 6 am in OSA patients, potentially associated with reduced larger VWFMs together with decreased VWF antigen levels. Severe OSA may contribute to an arterial pro-thrombotic state.

KEYWORD: ADAMTS13, obstructive sleep apnea, von Willebrand factor

Obstructive sleep apnea (OSA) is characterized 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, hyperlipidemia, 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 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 it has not been evaluated on a basis of arterial thrombosis generated under high shear stress in microvasculatures, where von Willebrand factor (VWF) plays a critical role.

VWF is a macromolecular plasma protein, exclusively produced in and released from vascular endothelial cells, and exerts pivotal effects on both hemostasis and thrombosis. VWF assembles into unusually large VWF multimers (UL-VWFMs) consisting of identical 250 kDa subunits, before it is released 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 hemostasis 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 thrombocytopenic purpura (TTP), a life-threatening generalized disease [8-11].

It is now well established that high plasma levels of VWF antigen (VWF:Ag) are linked to increased risk for ischemic heart disease and ischemic stroke [12-14]. Further, 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 upregulating VWF expression,

and indirectly via autocrine and paracrine signaling downstream of hypoxia-induced inflammatory cytokines including interleukin-6 (IL-6), IL-8, and tumor 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 analyzed 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 OSA patient plasmas taken at dawn are correlated with the clinical severity of apneic episodes.

PATIENTS, MATERIALS, and METHODS

Patients

Between Feb 2004 and Apr 2011, 284 patients received full standard diagnostic polysomnography (PSG) at Nara Medical University Hospital. Among them, 86 patients were diagnosed with normal or mild OSA (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 about these 58 patients is shown in Supplementary Table 1. 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). As sleep controls, 25 healthy volunteers (88% male) who had undergone PSG studies were also enrolled.

Blood sampling

Plasma samples were collected from OSA patients at three time points; at 9 pm before PSG, at 6 am after the PSG without CPAP, and at 6 am after CPAP treatment. From sleep control subjects, plasma samples were collected at 6 am. Blood was collected in plastic

tubes containing 1/10th volume of 3.8% Na3-citrate as an anticoagulant, and platelet-poor plasma was prepared by centrifugation at 3,000g for 15 minutes at 4°C. Aliquots were stored at –80°C prior to use. To obtain platelet counts, blood was collected into tubes containing EDTA as an anticoagulant and analyzed with a Coulter counter.

Sleep Study

Polysomnography (PSG) was performed using a computerized polysomnography system (Alice 4; Respironics; Pitteburgh, PA, USA). Data acquisition began at 9:00 pm and continued until 6:00 am the following morning. Apnea was defined as a cessation of airflow for 10 seconds or more, and hypopnea was defined as a decrease in airflow at least 50% for a minimum of 10 seconds or a clear decrease in airflow (>20%) followed by either oxygen desaturation of more than 3% or signs of physiological arousal. The apnea-hypopnea index (AHI) was calculated as the number of apnea-hypopnea events per hour of total sleeping time. We also calculated the ODI (oxygen desaturation index), 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; Pittsburgh; PA; USA), with PSG monitoring. Apneic episodes were substantially reduced or eliminated during treatment with nasal CPAP.

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

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, 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].

VWFM were analyzed by SDS-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). Multimers were classified as low molecular weight (LMW-VWFM; corresponding to bands 1−5 in VWFM analysis), intermediate molecular weight (IMW-VWFM; bands 6−10), and high molecular weight (HMW-VWFM; bands 11 and higher) [24]. High molecular weight bands that were not detected in normal plasma (NP) were defined as UL-VWFM. The

levels of LMW-, IMW-, HMW-VWFM were calculated using NIH ImageJ. For quantitative analyses, we calculated the ratios of the densities of VWFM, LMW, IMW, and HMW relative to total VWF multimer density. Further, multimeric VWF:Ag levels were calculated by multiplying VWF:Ag level by the LMW, IMW, and HMW ratios.

The plasma VWF collagen binding activity (VWF:CB) was measured using an enzyme immunoassay using a commercially available kit (VWF:CB ELISA, PROGEN BIOTECHNIK, GMBH, 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 means ± SD. Comparisons between OSA patients and controls were analyzed using the 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 analyzed using the Mann−Whitney U-test or chi-square test. All analyses were carried out using StatView (SAS Institute Cary, NC, USA). A p value less than 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. Eighteen, seven, and four patients in the OSA group were being treated for hypertension, hyperlipidemia, 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 SpO_2 (%).

Plasma VWF:Ag levels at 6 am were significantly lower in patients with OSA compared to controls, but plasma ADAMTS13:AC at 6 am did not differ between these groups. Interestingly, the plasma ADAMTS13:AC at 6 am in both OSA patients and sleep controls were lower than those of above mentioned healthy controls ($p<0.01$).

Chronological changes of plasma VWFM patterns categorize the patients with OSA into 3 groups

We analyzed VWFM patterns in plasmas taken from OSA patients, obtained at 9 pm and 6 am following sleep with or without CPAP. Based on these results, we categorized 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 sleep controls (n=6). Patients in Group 2 (n=18) exhibited reduced HMW-VWFM at 9 pm and persistent UL-VWFM at 6 am, without or with CPAP. Patients in Group 3 (n=11) had normal VWFM patterns at 9 pm, reduced predominantly HMW-VWFM at 6 am without CPAP, and returned to a normal VWFM pattern after CPAP therapy.

The decrease in HMW-VWFM and concomitant increase in LMW-VWFM could reflect either enhanced proteolysis by ADAMTS13 or extensive consumption secondary to platelet aggregation. Therefore, we first calculated the ratio of LMW-VWFM to total VWFM (LMW ratio) at 6 am without CPAP (Fig. 2A), and subsequently determined the relationship between LMW ratio and AHI. As shown in Fig. 2B, these two parameters are significantly correlated ($p<0.05$), suggesting that the degree of hypoxia during apneic events is related to VFWM processing and/or consumption.

Chronological changes of plasma levels of VWF:Ag, VWFM ratio, and ADAMTS13:AC in 3 patient groups with OSA

Plasma levels of VWF:Ag at 9 pm, 6 am without CPAP, and 6 am with CPAP were determined in all three groups of OSA patients. As shown in Fig. 3 (top), plasma VWF:Ag levels were almost unchanged in Group 1 patients, but significantly increased between 9 pm and 6 am in Group 2 patients. Notably, however, VWF:Ag levels remarkably decreased between 9 pm and 6 am in Group 3.

We then determined levels of HMW, IMW, and LMW in all three groups. In Group 1, HMW-VWFM was slightly increased at 6 am with CPAP, relative to 6 am without CPAP. In Group 2, HMW-VWFM significantly increased at 6 am compared to 9 pm, confirming the results of the VWFM analysis used for defining Groups 1−3. Consistent with this, in Group 3, the IMW-VWFM at 6 am was significantly lower than that at 9 pm; CPAP treatment reversibly increased the HMW-VWFM at 6 am, in accordance with the increase in plasma VWF:Ag level.

In contrast, no changes in the plasma ADAMTS13:AC were seen at 9 pm, 6 am, or 6 am 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 6 am without CPAP were inversely correlated with the LMW ratio ($p<0.01$), as shown in Fig. 4A. Furthermore, as shown in Fig. 4B, plasma levels of VWF:CB at 6 am were 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 6 am in Group 3 patients.

Decreased platelet counts at dawn in the untreated patients with OSA

A pair of platelet counts at 9 pm and 6 am without CPAP was determined in 31 of 58 OSA patients and in 6 of 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 hematocrit. The ratios in sleep controls did not exhibit significant changes between 9 pm and 6 am (Fig. 5A), whereas they were lower at 6 am in untreated OSA patients ($p<0.01$) (Fig. 5B). However, none of the patients who received CPAP treatment developed overt clinical signs of thrombotic complications. These results

suggest that in untreated OSA patients, albeit asymptomatic but platelet consumption to be a lesser extent might occur during sleep.

Patient characteristics of Groups 1, 2, and 3

Table 2 summarizes the demographic and measured parameters of OSA patients categorized 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 VWF multimers at dawn (6 am), represent the highest severity of OSA among the three groups.

Consistent with these results, decreased plasma levels of VWF:Ag in two time interval ([6 am] − [9 pm]) was remarkable in Group 3, in comparison to those in Groups 1 and 2. Interestingly, the differences of LMW ratio in two times ([6 am] − [9 pm]) was significantly higher in Group 3 than those of Group 1 or Group 2. These results indicated that decreased VWF:Ag at 6 am was caused primarily by the reduction in larger VWFMs. On the other hand, no significant change in ADAMTS13:AC between two times ([6 am] − [9 pm]) was observed in Group 3, whereas such a change was observed in Groups 1 and 2, leaving the physiological relevance unaddressed.

Relationship of AHI and Groups 1~3 of VWF multimer patterns in OSA patients

AHI is an excellent means showing OSA severity, to which here we have categorized into 3 groups: AHI 15~<30 (moderate), 30 ~ <60 (severe), and ≥60 (extremely severe). As shown in Table 3, OSA patients with Group 1 and 2 consisted of those with variable AHI levels. Notably, however, none of OSA patients with Group 3 had AHI 15~<30, and they uniformly had AHI ≥30 and more predominantly with AHI ≥60. The incident of Group 1 was lower in AHI groups of 30 ~ <60 and 60~ than those of 15 ~ <30 ($p<0.05$). In contrast, the incident of Group 3 was higher in AHI group of 60 ~ than those of 15 ~ <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 age 40 in normal individuals; by the age of 60, they may reach approximately 120−140% of the healthy normal baseline [26]. The mean age of OSA patients enrolled in this study was 44.7 years old, whereas that of control subjects was 38.3 years old. However, the plasma VWF:Ag levels collected at 6 am were significantly lower for OSA patients than for control subjects (Table 1). In contrast, plasma ADAMTS13 activity decreases after age 40 in normal individuals [27]. Among our study patients and controls, plasma ADAMTS13:AC was lower than in healthy controls aged 20−40 years ($p<0.01$), 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 analyzed VWFM patterns chronologically at three time points: at 9 pm, and at 6 am with or without overnight CPAP treatment. As expected, a majority of OSA patients (29/58, 50%) had consistently normal VWFM patterns, categorized as Group 1. Two smaller groups of patients had increased UL- and HMW-VWFM (18/58, 31%) or decreased UL- and HMW-VWFM (11/58, 19%) at 6 am; these were categorized 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 hypothesized that the elevated LMW ratio seen in our OSA patients was likely due to 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 categorized as Group 3. Although the mechanism is not yet fully elucidated, the highly multimerized VWF 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 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 9 pm and 6 am. This decrease was associated with reductions in both the plasma VWF:Ag levels and HMW-VWFM in Group 3. Quantitative analyses of VWF multimers in Group 3 showed that levels of HMW-VWFM increased significantly after CPAP treatment, compared with measurements taken at 6 am without CPAP. This is consistent with low-level consumption of UL- and HMW-VWFM 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 apnea in OSA patients. The results obtained by VWF multimer 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 6 am 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, the large-scale studies, together with chronological

measurement of platelet counts and VWF:CB, would be the focus in the following studies.

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STATEMENT OF INTEREST
None declared.

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REFERENCES

1. Malhotra A, White DP. Obstructive sleep apnoea. Lancet 2002; 360: 237-245.
2. Mooe T, Rabben T, Wiklund U, et al. Sleep-disordered breathing in men with coronary artery disease. Chest 1996;109:659–663.
3. Shahar E, Whitney CW, Redline S, et al. Sleep-disordered breathing and cardiovascular disease: cross-sectional results of the Sleep Heart Health Study. Am J Respir Crit Care Med 2001;163:19-25.
4. Redline S, Yenokyan G, Gottlieb DJ, et al. Obstructive sleep apnea-hypopnea and incident stroke: the sleep heart health study. Am J Respir Crit Care Med 2010;182:269-277.
5. Marin JM, Carrizo SJ, Vicente E, et al. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. Lancet 2005; 365: 1046-1053.
6. Epstein MD, Segal LN, Ibrahim SM, et al. Snoring and risk of obstructive sleep apnea in patients with pulmonary embolism. Sleep 2010;33:1069-1074.
7. Bosanquet JP, Bade BC, Zia MF, et al. Patients with venous thromboembolism appear to have higher prevalence of obstructive sleep apnea than the general population. Clin Appl Thromb Hemost 2010;[Epub ahead of print]
8. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost 2003; 1: 1335-1342.
9. Sadler JE, Moake JL, Miyata T, et al. Recent advances in thrombotic thrombocytopenic purpura. Hematology Am Soc Hematol Educ Program 2004:407-423.
10. Moake JL, Rudy CK, Troll JH, et al. Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med 1982; 307: 1432-1435.
11. Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature 2001; 413: 488-494.
12. Thompson SG, Kienast J, Pyke SD, et al. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. N Engl J Med 1995; 332: 635-641.

13. Chion CK, Doggen CJ, Crawley JT, et al. ADAMTS13 and von Willebrand factor and the risk of myocardial infarction in men. Blood 2007; 109: 1998-2000.
14. Bongers TN, de Maat MP, van Goor ML, et al. High von Willebrand factor levels increase the risk of first ischemic stroke: influence of ADAMTS13, inflammation, and genetic variability. Stroke 2006; 37: 2672-2677.
15. Kaikita K, Soejima K, Matsukawa M, et al. Reduced von Willebrand factor-cleaving protease (ADAMTS13) activity in acute myocardial infarction. J Thromb Haemost 2006; 4: 2490-2493.
16. Wilkie ME, Stevens CR, Cunningham J, et al. Hypoxia-induced von Willebrand factor release is blocked by verapamil. Miner Electrolyte Metab 1992; 18: 141-144.
17. Pinsky DJ, Naka Y, Liao H, et al. Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. J Clin Invest 1996; 97: 493-500.
18. von Kanel R, Loredo JS, Ancoli-Israel S, et al. Association between sleep apnea severity and blood coagulability: Treatment effects of nasal continuous positive airway pressure. Sleep Breath 2006; 10: 139-146.
19. Robinson GV, Pepperell JC, Segal HC, et al. Circulating cardiovascular risk factors in obstructive sleep apnoea: data from randomised controlled trials. Thorax 2004; 59: 777-782.
20. Bartlett A, Dormandy KM, Hawkey CM, et al. Factor-VIII-related antigen: measurement by enzyme immunoassay. Br Med J 1976; 1: 994-996.
21. Matsumoto M, Kawaguchi S, Ishizashi H, et al. Platelets treated with ticlopidine are less reactive to unusually large von Willebrand factor multimers than are those treated with aspirin under high shear stress. Pathophysiol Haemost Thromb 2005; 34: 35-40.
22. Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. J Clin Invest 1980; 65: 1318-1325.
23. Budde U, Schneppenheim R, Plendl H, et al. Luminographic detection of von Willebrand factor multimers in agarose gels and on nitrocellulose membranes. Thromb Haemost 1990; 63: 312-315.

24. Veyradier A, Nishikubo T, Humbert M, et al. Improvement of von Willebrand factor proteolysis after prostacyclin infusion in severe pulmonary arterial hypertension. Circulation 2000; 102: 2460-2462.
25. Kato S, Matsumoto M, Matsuyama T, et al. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. Transfusion 2006; 46: 1444-1452.
26. Conlan MG, Folsom AR, Finch A, et al. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. Thromb Haemost 1993; 70: 380-385.
27. Kokame K, Nobe Y, Kokubo Y, et al. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol 2005; 129: 93-100.

Table 1. Characteristics of patients with OSA and sleep controls

	OSA (n=58)	Sleep controls (n=25)	p value
Number (Male/Female)	58(55/3)	25(22/3)	NS
ABO Blood type(A/B/O/AB)	18/8/26/6	12/2/8/3	NS
Age (yr)	44.7 ± 9.9	38.3 ± 7.1	<0.01
Body Mass Index (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 SpO_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 6 am	103.1 ± 61.4	143.5 ± 63.8	<0.01
ADAMTS13:AC levels (%) at 6 am	56.8 ± 22.6	61.7 ± 20.6	NS

Data are presented as mean±SD
OSA : obstructive sleep apnea, AHI : apnea hypopnea index, ODI3 : 3% oxygen desaturation index, SpO_2 : oxygen saturation
VWF:Ag : von Willebrand factor antigen, ADAMTS13:AC : ADAMTS13 activity
NS : not significant

Table 2. Characteristics and differences of parameter between 9 pm and 6 am of patients with OSA in Group 1-3

	Group 1	Group 2	Group 3	Overall p value
Number (Male/Female)	28/1	18/0	9/2	NS
Blood type (A/B/O/AB)	7/4/14/4	4/4/7/3	7/0/4/0	NS
Age (yr)	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[a]
ODI3	35.7 ± 18.2	44.1 ± 19.3	53.2 ± 21.5	<0.01[b]
Differences in two time interval [6am]-[9pm]				
VWF:Ag (%)	2.1 ± 34.8	10.8 ± 22.0	-28.1 ± 40.6	<0.05[a]
LMW ratio (%)	-0.27 ± 5.24	-4.46 ± 8.69	16.69 ± 16.92	<0.01[c]
ADAMTS13:AC (%)	4.4 ± 13.1	-8.5 ± 25.9	2.4 ± 21.4	<0.05[d]
Plt/Ht (10^9/L/%)	-0.045 ± 0.036 (n=15)	-0.034 ± 0.038 (n=10)	-0.043 ± 0.029 (n=6)	NS

Data are presented as mean±SD,
a:<0.05 between Group 1, 2 and 3 b:<0.01 between Group 1 and 3
c:<0.01 between Group 1, 2 and 3 d:<0.05 between Group 1 and 2
OSA : obstructive sleep apnea, AHI : apnea hypopnea index, ODI3 : 3% oxygen desaturation index, SpO_2 : oxygen saturation
VWF:Ag : von Willebrand factor antigen, ADAMTS13:AC : ADAMTS13 activity , Plt/Ht: the ratio of platelet count to hematocrit
NS: not significant

Table 3. Characteristics and thrombotic parameters of patients classified with AHI

	15≤AHI<30 (n=15)	30≤AHI<60 (n=22)	60≤AHI (n=21)	Overall p value
Number (Male/Female)	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[a]
VWF multimer Group				
Group 1	12 (80 %)	8 (36 %)	9 (43 %)	<0.05[b]
Group 2	3 (20 %)	10 (45 %)	5 (24 %)	NS
Group 3	0	4 (18 %)	7 (33 %)	<0.05[c]
VWF:Ag at 6 am(%)	98.5 ± 49.1	98.5 ± 55.7	111.3 ± 75.5	NS
ADAMTS13:AC at 6 am (%)	58.1 ± 20.2	55.2 ± 21.9	57.6 ± 25.6	NS
VWF:CB at 6 am(U/ml)	1.29 ±0.39 (n=13)	1.23 ± 0.50 (n=19)	1.09 ± 0.38 (n=19)	NS
Plt/Ht at 6 am (10^9/L/%)	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,
a:<0.01 between all AHI groups, b:<0.05 between 15≤AHI<30 and 30≤AHI<60, 60≤AHI
c:<0.05 between 15≤AHI<30 and 60≤AHI
OSA : obstructive sleep apnea, AHI : apnea hypopnea index ODI3 : 3% oxygen desaturation index
VWF:Ag : von Willebrand factor antigen ADAMTS13:AC : ADAMTS13 activity
NS: not significant

Figure legends

FIGURE 1. Patterns of VWFM correspond to three patient groups.

OSA patients were categorized into three groups based on the results of VWFM analysis, using sequential samples. Representative results from each group are shown. The patients in Group 1 showed a consistently normal pattern of VWFM. The patients in Group 2 had increased unusually large (UL)- and high molecular weight (HMW)-VWFM at 6 am compared to 9 pm. Patients in Group 3 had decreased UL- and HMW-VWFM at 6 am compared to 9 pm.

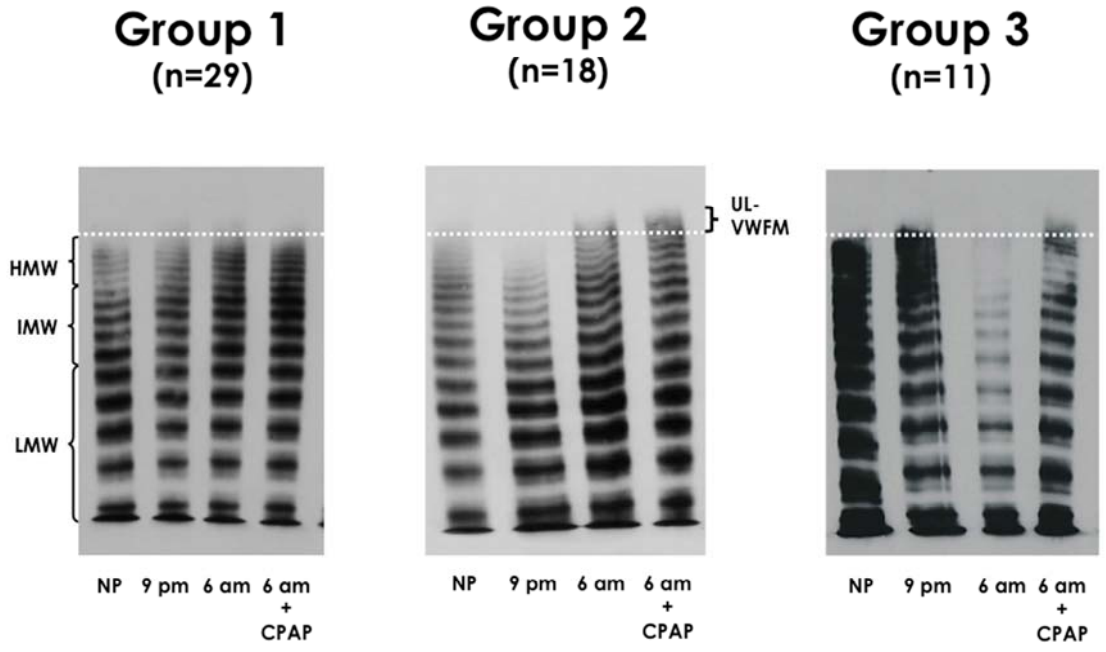

Fig. 1

FIGURE 2. Relationship between LMW ratio and hypoxia.

A. Quantitative analysis of VWFM was performed by calculating the density of LMW-VWFM relative to total multimer density (LMW ratio). A representative result of VWF analysis at 6 am is shown.

B. The LMW ratio of OSA patients was significantly correlated to AHI ($p < 0.05$).

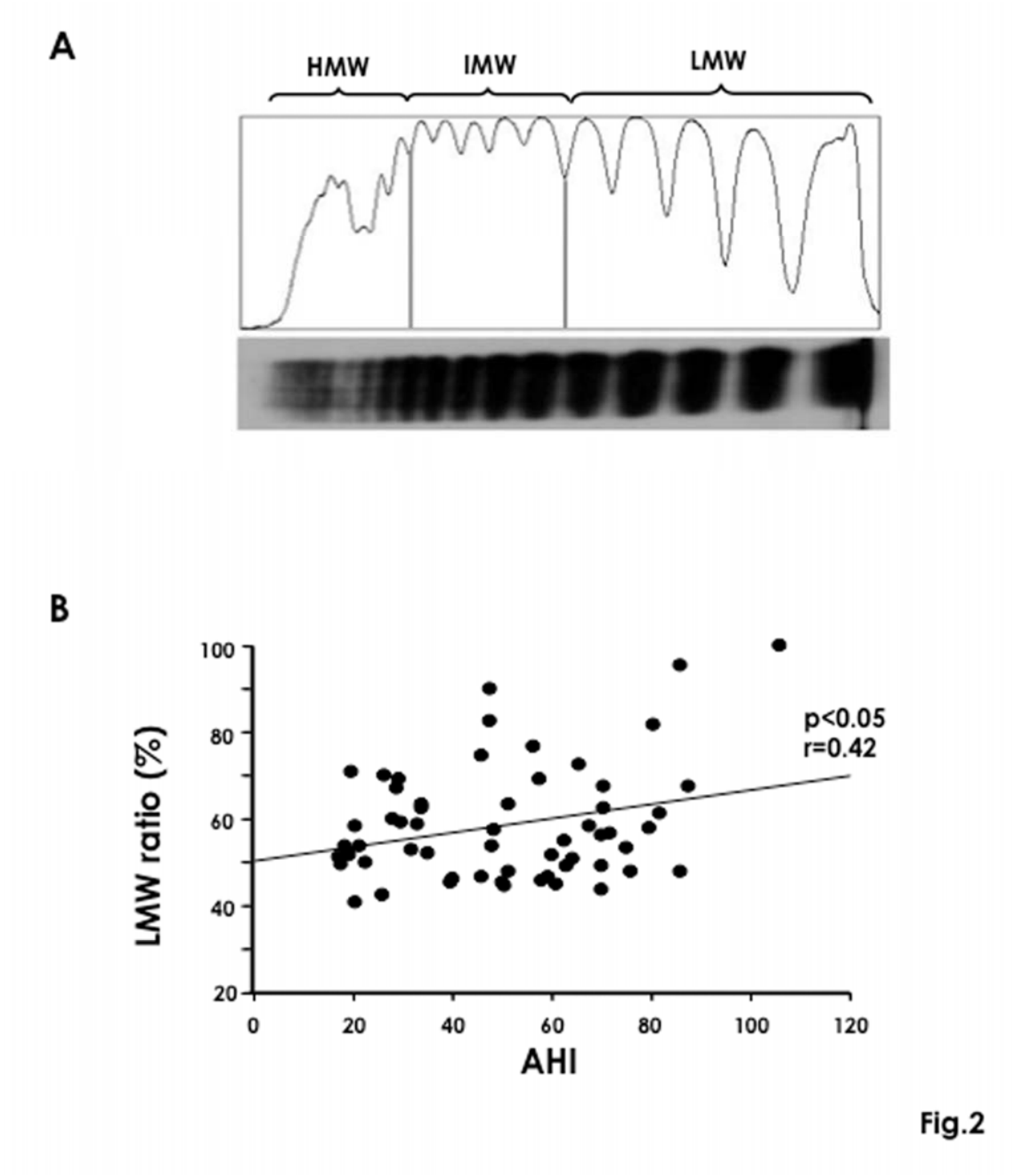

Fig.2

FIGURE 3. Changes in serial VWF:Ag levels and ADAMTS13:AC in Groups 1-3

VWF:Ag levels were divided into HMW-, IMW-, LMW-VWFM groups by multiplying the VWF:Ag level by the results of the multimeric analyses. Groups were first compared using the Kruskal−Wallis H test; significantly different groups were then analyzed using the Mann−Whitney U-test. * p<0.05, ** p<0.01

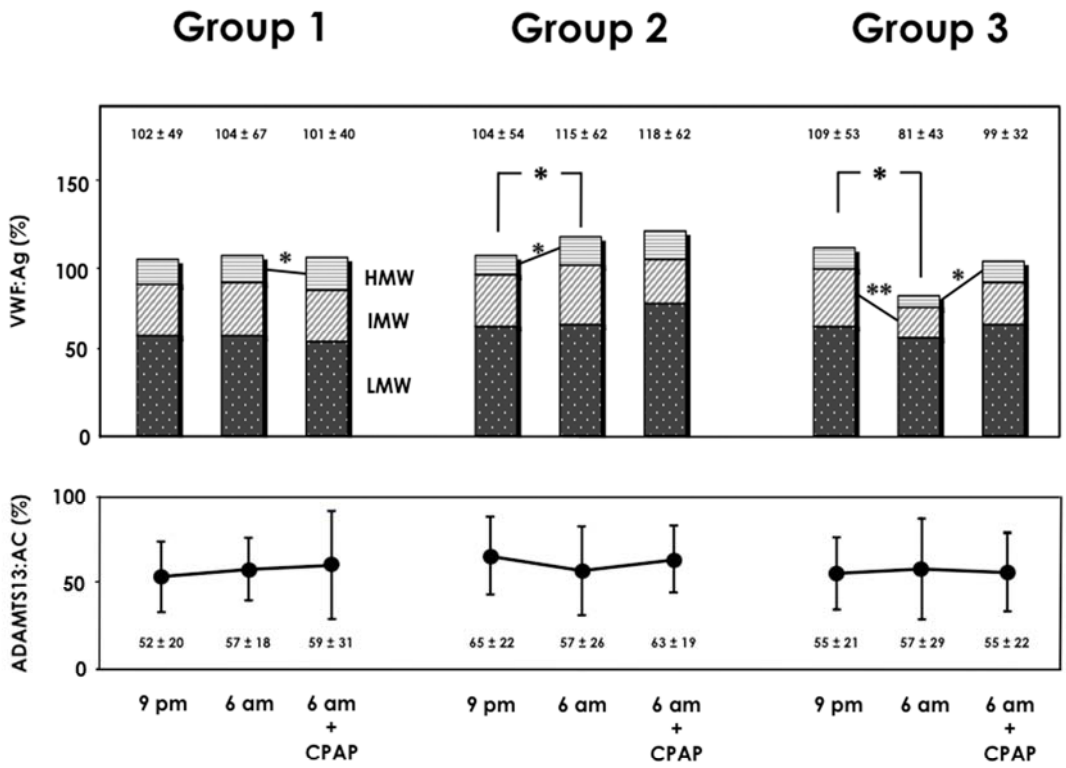

Fig.3

FIGURE 4. Relationship VWF:CB and LMW ratio, and comparison of VWF:CB at 6 am in each group.

VWF:CB was measured in 53 out of 58 OSA patients.

A. Significant inverse correlation between LMW ratio and VWF:CB at 6 am in OSA patients.

B. VWF:CB at 6 am in Group 3 was significantly lower than in Groups 1 and 2.

*p<0.05

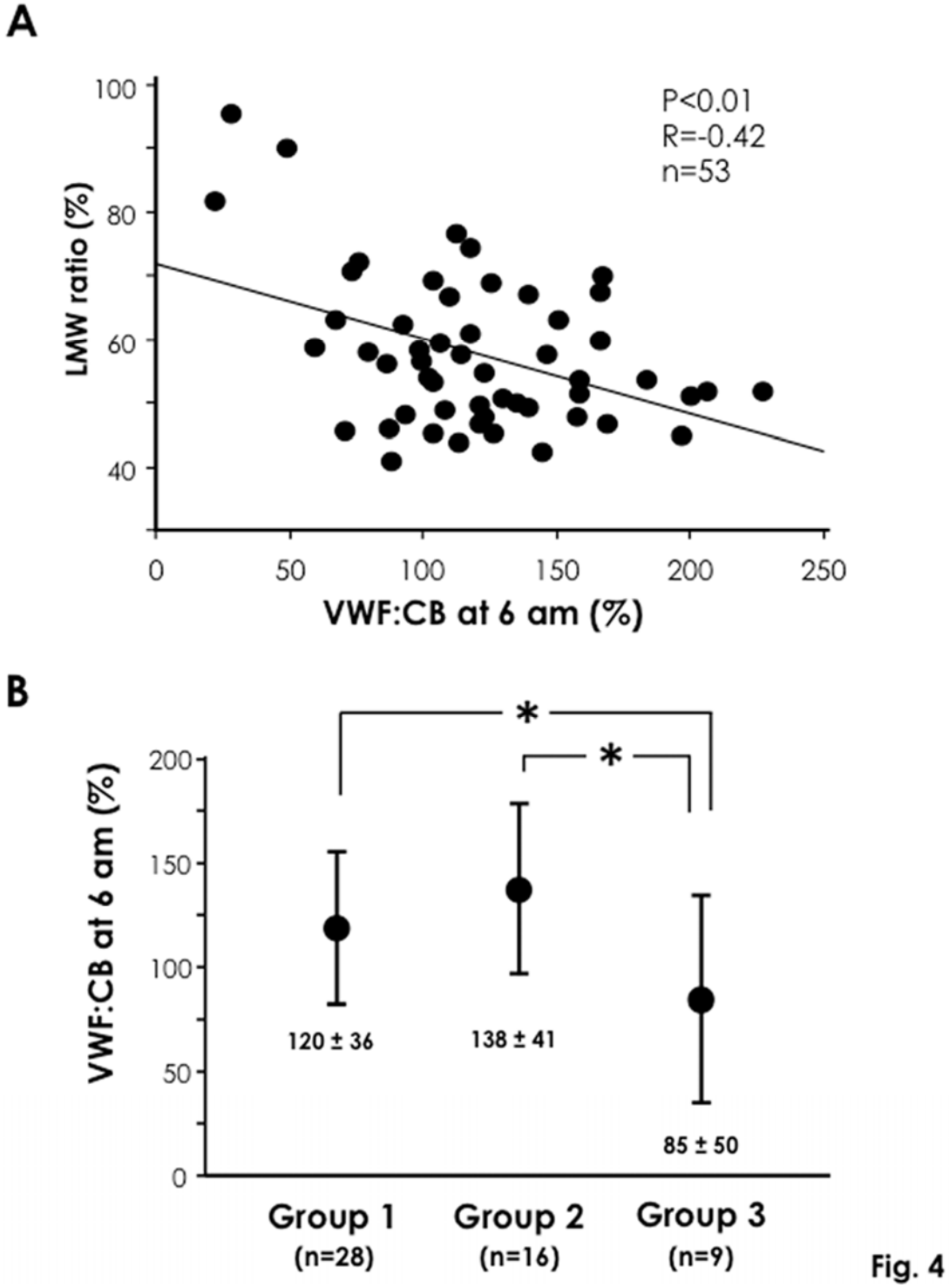

FIGURE 5. Overnight platelet counts to hematocrit ratios decreased in patients with OSA.

Platelet counts were normalized to the patient's hematocrit to control for differences in hydration status. Ratios of platelet count to hematocrit were obtained at 9 pm and 6 am in 6 sleep controls (A) and in 31 OSA patients without CPAP treatment.

A. In sleep controls, the ratios did not change between time points.

B. In OSA patients, the ratio exhibited significant changes between time points.

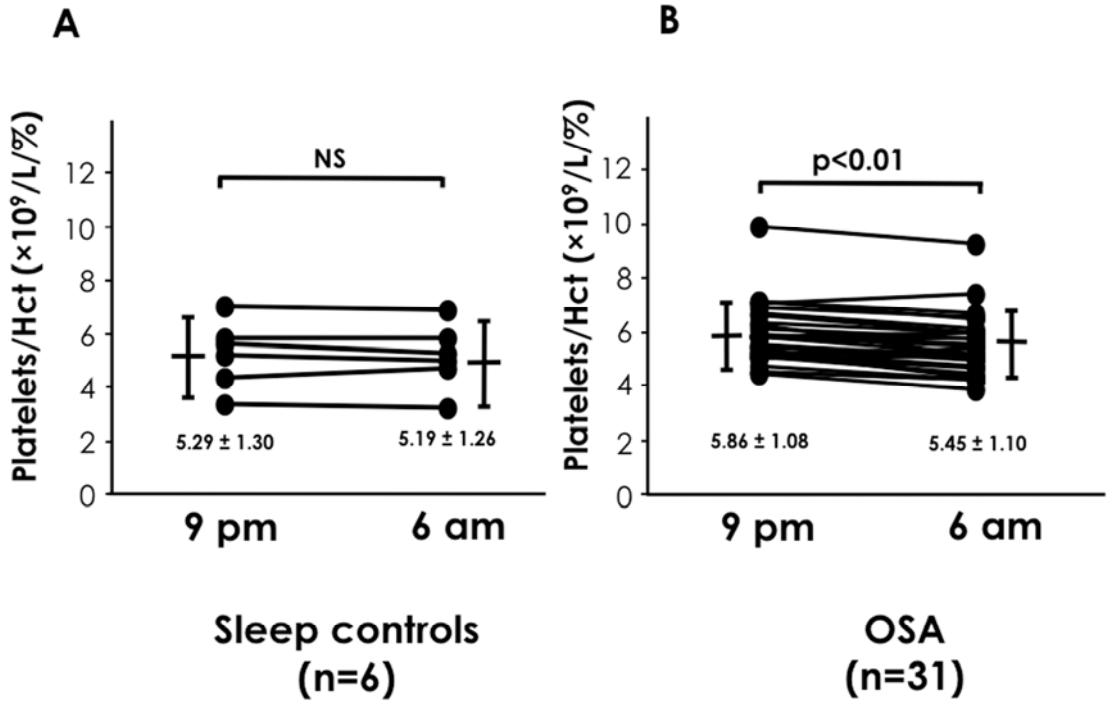

Fig. 5