Supplemental Material

Complement Promotes Endothelial von Willebrand Factor and Angiopoietin-2 Release in Obstructive Sleep Apnea: von Willebrand Factor and Angiopoietin-2 Release in Sleep Apnea

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Assessment of plasma vWF multimer levels

Analysis of von Willebrand Factor multimeric patterns in human plasma was performed in a commercial laboratory (ARUP) [1, 2]. Sodium citrate plasma was treated with an anionic detergent to convert the proteins to anionic detergent-protein complexes. In these complexes, the native conformation of proteins is disrupted and they all assume the same conformation and the same negative charge per mass unit. These complexes are applied to a concentrated agarose gel where electrophoresis separates the complexes according to their molecular weight. The vWF multimers with molecular weight between 500 and 20,000 kDa are separated and immunoprecipitated with a specific anti-von Willebrand factor antiserum. The different bands are then visualized with a peroxidase-labeled antibody and a specific substrate.

Supplemental references:

1. Flood VH, Christopherson PA, Gill JC, Friedman KD, Haberichter SL, Bellissimo DB, Udani RA, Dasgupta M, Hoffmann RG, Ragni MV, Shapiro AD, Lusher JM, Lentz SR, Abshire TC, Leissinger C, Hoots WK, Manco-Johnson MJ, Gruppo RA, Boggio LN, Montgomery KT, Goodeve AC, James PD, Lillicrap D, Peake IR and Montgomery RR. Clinical and laboratory variability in a cohort of patients diagnosed with type 1 VWD in the United States. *Blood*. 2016;127:2481-2488.
2. Jacobi PM, Gill JC, Flood VH, Jakab DA, Friedman KD and Haberichter SL. Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood*. 2012;119:4543-4553.

Table S1. Characteristics of the Participants.

	OSA patients (n=109)	**Control subjects** (n=67)	**p value**
Age (years)	44±13	38±13	0.003
Gender (% female)	53	67	NS
Body mass index (kg/m^2)	37.4±10.2	32.15±9.7	<0.001
Apnea-hypopnea index (events/h of sleep)	24.7±26.4	1.53±1.50	<0.001
Systolic blood pressure (mmHg)	127±16.1	121.7±13.7	0.03
Diastolic blood pressure (mmHg)	79±10.1	77.6±8.76	NS
Oxygen Desaturation Index (events/h of sleep)	16.7±25.3	0.61±1.03	<0.001
Epworth	10.13±5.76	7.85±4.71	0.008
SaO_2 nadir %	82.12±13.47	90.2±5.98	<0.001
t<SaO_2 90% (% of the total sleep time)*	4.0±12.35	0.14±0.41	0.003
Hypertension, n (%)	30 (28)	9 (14)	NS

OSA= Obstructive Sleep Apnea

SaO_2= Arterial Oxyhemoglobin Saturation

*Time spent below SaO_2 of 90% during sleep

Data are presented as mean±SD and %

NS=not significant

Table S2. Bivariate association with angiopoietin-2.

	Estimate	Std. Error	t value	p-value
AHI	10.492	8.738	1.201	0.233
Age	4.415	12.305	0.359	0.721
Gender	292.014	320.360	0.912	0.364
BMI	25.689	14.100	1.822	0.072
SBP	-0.061	10.672	-0.006	0.995
DBP	-19.427	17.707	-1.097	0.276

AHI= Apnea-hypopnea index (events/h of sleep)

BMI= Body mass index (kg/m^2)

SBP= Systolic blood pressure (mmHg)

DBP= Diastolic blood pressure (mmHg)

Table S3. Correlation between intermediate molecular weight (IMW) von Willebrand factor multimer levels and apnea-hypopnea index (r^2 = 0.3115) adjusted for gender and diastolic blood pressure.

	Estimate	Std. Error	t value	p-value
(Intercept)	15.3898	15.0010	1.0259	0.3124
AHI	0.3015	0.1094	2.7566	0.0094
Gender	10.2831	3.6273	2.8349	0.0078
DBP	0.3974	0.1545	2.5715	0.0148

AHI= Apnea-hypopnea index (events/h of sleep)

DBP= Diastolic blood pressure (mmHg)

Table S4. Correlation between low molecular weight (LMW) von Willebrand factor multimer levels and apnea-hypopnea index (r^2 = 0.3085) adjusted for body mass index.

	Estimate	Std. Error	t value	p-value
(Intercept)	0.8932	7.0597	0.1265	0.9000
AHI	0.3116	0.1422	2.1916	0.0347
BMI	0.5470	0.1714	3.1908	0.0029

AHI= Apnea-hypopnea index (events/h of sleep)

BMI= Body mass index (kg/m^2)

Table S5. Correlations between low molecular weight (LMW) von Willebrand factor multimer levels and obstructive sleep apnea severity parameters.

Correlations	Estimate coefficients	Std. Error	p-value	r^2
Unadjusted AHI	0.3558	0.1577	0.0299	0.1183
AHI after adjusting for BMI	0.3116	0.1422	0.0347	0.3085
Unadjusted ODI	0.7429	0.2673	0.0113	0.2683
ODI after adjusting for BMI	0.6259	0.2524	0.0222	0.4078
Unadjusted t < SaO_2 90%	8.407	2.906	0.0071	0.2237
t < SaO_2 90% after adjusting for BMI	6.5886	2.7506	0.0235	0.3729

AHI= Apnea-hypopnea index (events/h of sleep)

BMI= Body mass index (kg/m^2)

ODI= Oxygen desaturation index (events/h of sleep)

t < SaO_2 90%= Time spent below SaO_2 of 90% during sleep

Table S6. Bivariate associations with CD59-vWF Duolink levels.

	Estimate	Std. Error	t value	p-value
AHI	0.083	0.109	0.764	0.452
Age	-0.040	0.128	-0.311	0.758
Gender	-0.485	3.162	-0.153	0.879
BMI	0.003	0.157	0.021	0.984
SBP	0.145	0.112	1.294	0.208
DBP	0.075	0.163	0.460	0.650

AHI= Apnea-hypopnea index (events/h of sleep)

BMI= Body mass index (kg/m^2)

SBP= Systolic blood pressure (mmHg)

DBP= Diastolic blood pressure (mmHg)

vWF = von Willebrand factor

Supplemental Figure Captions:

Figure S1. Circulating LMW vWF multimer levels in OSA. Quantitation of plasma LMW vWF multimers levels from OSA patients (*n* = 22: 45% female; age 40±13 years; BMI 42±14; AHI 18±18 events/h; ODI 8.3±13 events/h) and controls (*n* = 18: 83% female; age 35±11 years; BMI 36±10; AHI 1.1±1.3; ODI 0.6±0.6 events/h) expressed as band mean intensity (mean ± SD, permutation test).

vWF = von Willebrand factor; OSA = Obstructive Sleep Apnea; LMW = Low Molecular Weight; AHI = Apnea–Hypopnea Index; ODI = Oxygen Desaturation Index; BMI = body mass index.

Figure S2. Expression of CD59 on the cell surface and MAC deposition on EC in OSA. (A) Representative confocal images and quantitation of the cell surface CD59 expression in ECs harvested from OSA patients (*n* = 40) and controls (*n* = 19). EC plasma membrane is identified by immunofluorescence for VE-cadherin. (B) Representative confocal images and quantitation of MAC levels in ECs harvested from OSA patients (*n* = 40) and controls (*n* = 19). EC plasma membrane is identified by immunofluorescence for vascular endothelial (VE)-cadherin. All data throughout the figure are shown as the mean ± SE, 2-sided t-test.

EC = Endothelial Cell; MAC = membrane attack complex; OSA = Obstructive Sleep Apnea; VE = vascular endothelial.

Figure S3. IH effects on endothelial vWF and Ang-2 release in the absence of complement. (A) Quantitation of vWF levels in HUVECs complement-free culture media in normoxia and IH (*n* = 5) (ng/ml). (B) Quantitation of vWF expression on the cell surface in HUVECs cultured in complement-free media in normoxia and IH expressed as mean fluorescence intensity (*n* = 6). (C) Representative confocal images and quantitation of intracellular vWF protein expression in HUVECs cultured in complement-free media in normoxia and IH (*n* = 4). EC plasma membrane is identified by immunofluorescence for VE-cadherin. (D) Quantitation of mRNA levels in HUVECs cultured in complement-free media in normoxia and IH (*n* = 6). All data throughout the figure are shown as the mean ± SE, 2-sided t-test.

IH = Intermittent Hypoxia; vWF = von Willebrand factor; HUVECs = Human Umbilical Vein Endothelial Cells; DMSO = Dimethyl sulfoxide; VE = Vascular Endothelial.

Figure S4. Confirmation of HUVEC transfection with CD59 siRNA. Western blot probed with antibodies directed against CD59 and GAPDH. Transfection of HUVECs was carried out for 48 h using transfection reagent (Vehicle) and pooled siRNAs against CD59 [CD59 (966) siRNA to knock down CD59. A scrambled RNA (control RNA) served as a negative control. The expression of CD59 was almost completely knocked down by CD59 (966) siRNA.

HUVECs = Human Umbilical Vein Endothelial Cells; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Figure S5. Co-immunoprecipitation of VAMP3 and VAMP8 with CD59. Western blot probed with antibodies directed against CD59, VAMP3 and VAMP8 in the immunoprecipitates of VAMP3 and VAMP8 of HUVECs exposed to normoxia or IH. IgG served as negative control.

VAMP3 = Vesicle-associated Membrane Protein 3; VAMP8 = Vesicle-associated Membrane Protein 8; HUVECs = Human Umbilical Vein Endothelial Cells.

Figure S6. Intracellular calcium mediates IH-induced endothelial vWF and Angiopoietin-2 release after complement stimulation. (A) Representative confocal images of intracellular vWF protein expression in HUVECs after stimulation with recombinant C9 and treatment with BAPTA-AM or vehicle (0.1% DMSO) in normoxia and IH. EC plasma membrane is identified by immunofluorescence for VE-cadherin. (B) Quantitation of intracellular vWF protein expression (fluorescence area in μm^2) in HUVECs after stimulation with C9 and treatment with BAPTA-AM (*n* = 5) or vehicle (0.1% DMSO) (*n* = 8) in normoxia and IH. (C) Quantitation of vWF levels in HUVECs culture media after stimulation with C9 and treatment with BAPTA-AM or vehicle (0.1% DMSO) in normoxia and IH (*n* = 6). (D) Representative histogram with a log scale x-axis of vWF fluorescence intensity on the cell surface in HUVECs after stimulation with C9 and treatment with BAPTA-AM or vehicle (0.1% DMSO) in normoxia and IH. (E) Quantitation of vWF expression on the cell surface in HUVECs after stimulation with C9 and treatment with BAPTA-AM or vehicle (0.1% DMSO) in normoxia and IH, expressed as mean fluorescence intensity (*n* = 4). (F) Quantitation of angiopoietin-2 levels in culture media of HUVECs after stimulation with C9 and treatment with BAPTA-AM or

vehicle (0.1% DMSO) in normoxia and IH (*n* = 5). All data throughout the figure are shown as the mean ± SE, 2-sided t-test test.

IH = Intermittent Hypoxia; vWF = von Willebrand factor; HUVECs = Human Umbilical Vein Endothelial Cells; BAPTA-AM = 1, 2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester); DMSO = Dimethyl Sulfoxide; EC = Endothelial Cell; VE = Vascular Endothelial.

Figure S1

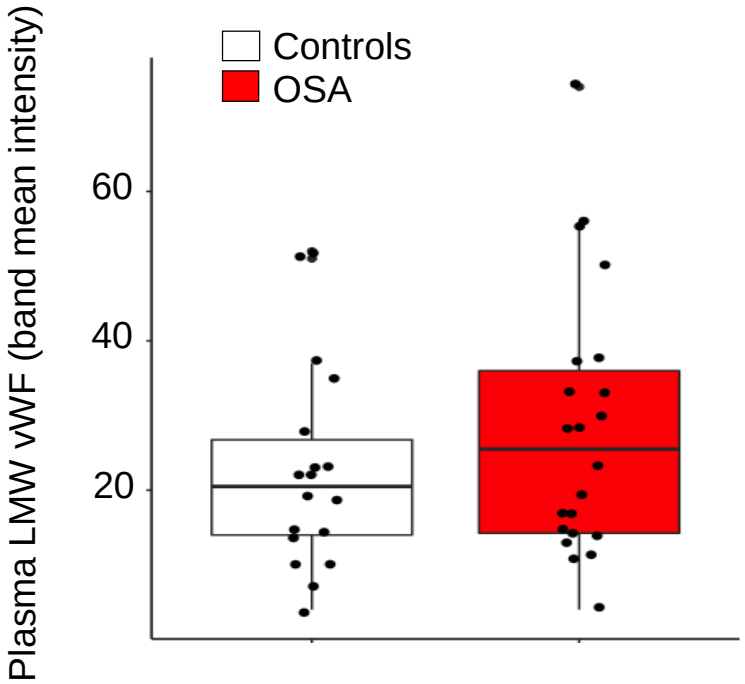


Figure S2

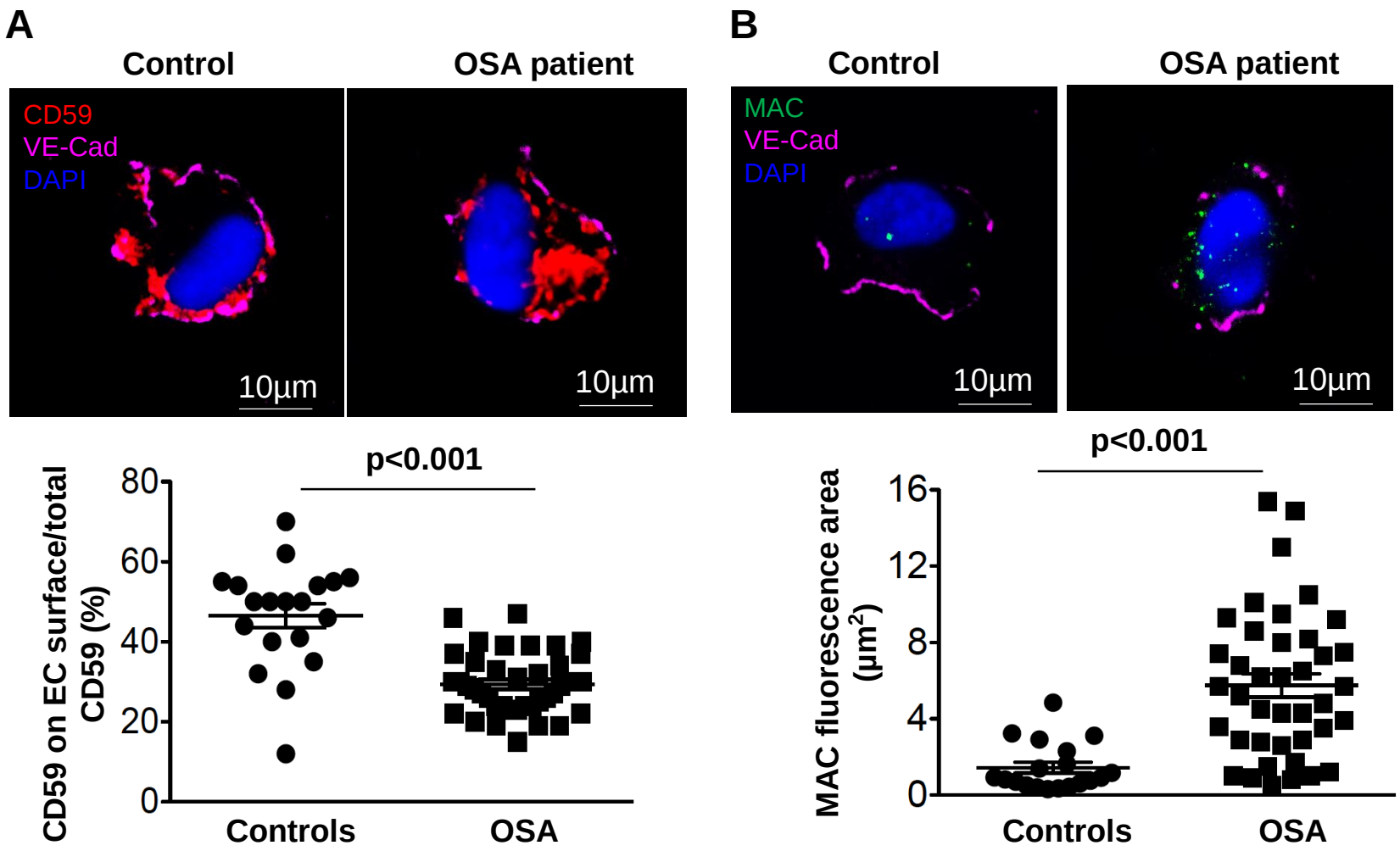

A
Control
OSA patient
CD59
VE-Cad
DAPI
10µm
10µm
p<0.001
CD59 on EC surface/total CD59 (%)
80
60
40
20
0
Controls
OSA
B
Control
OSA patient
MAC
VE-Cad
DAPI
10µm
10µm
p<0.001
MAC fluorescence area (µm²)
16
12
8
4
0
Controls
OSA

Figure S3

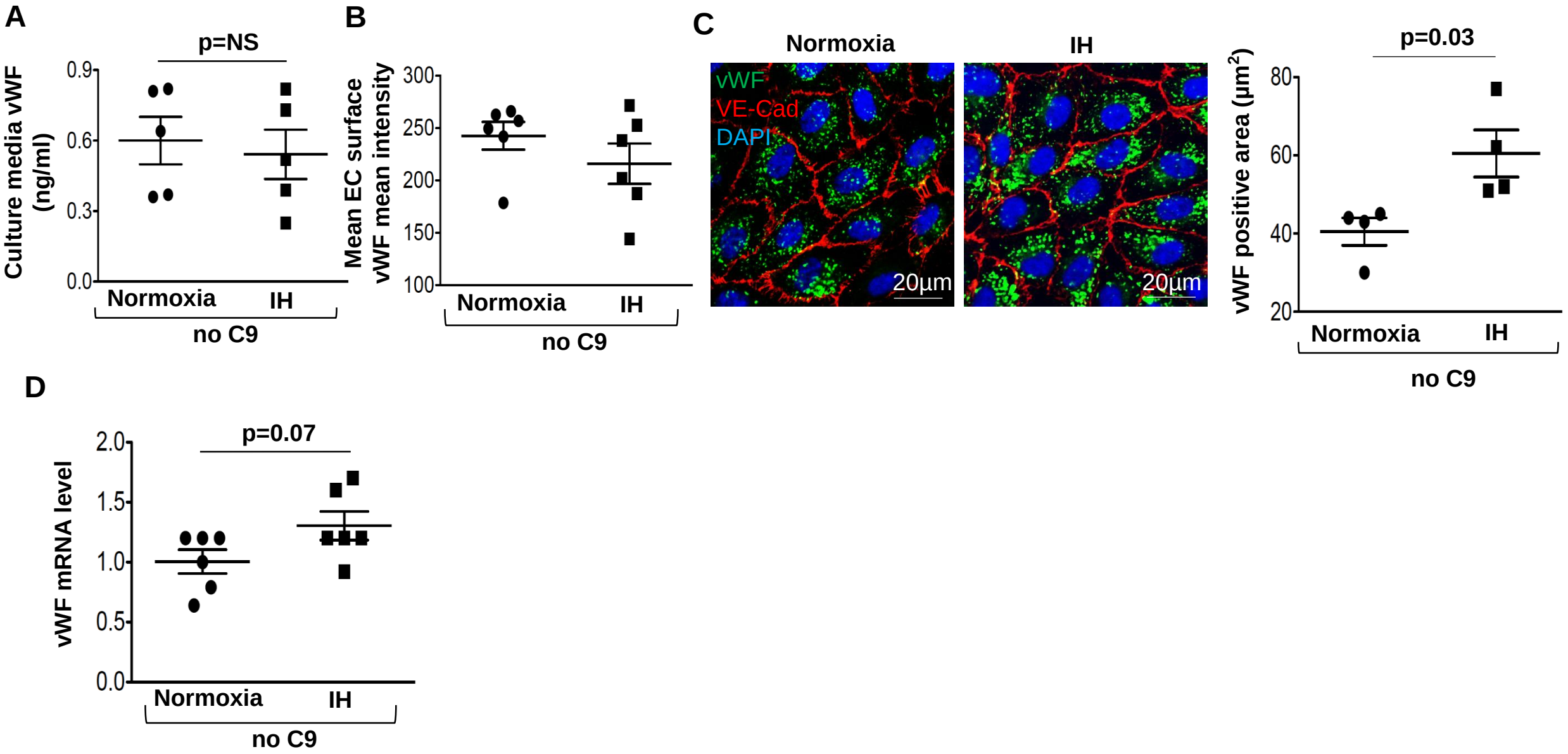

A
p=NS
Culture media vWF (ng/ml)
0.9
0.6
0.3
0.0
Normoxia
IH
no C9
B
Mean EC surface vWF mean intensity
300
250
200
150
100
Normoxia
IH
no C9
C
Normoxia
IH
vWF
VE-Cad
DAPI
20µm
20µm
p=0.03
vWF positive area (µm²)
80
60
40
20
Normoxia
IH
no C9
D
p=0.07
vWF mRNA level
2.0
1.5
1.0
0.5
0.0
Normoxia
IH
no C9

Figure S4

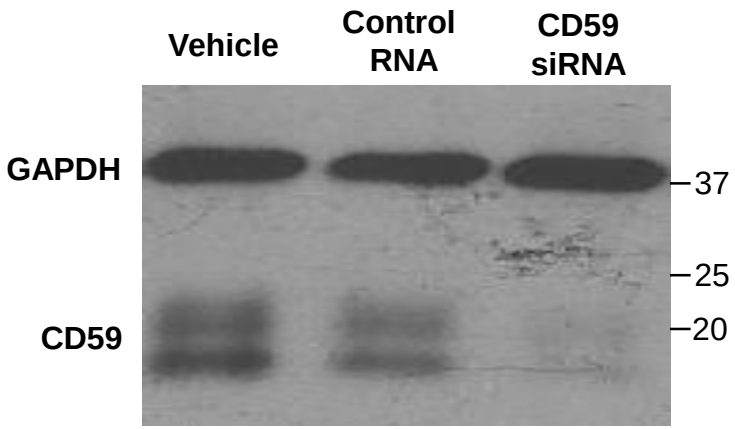


Figure S5

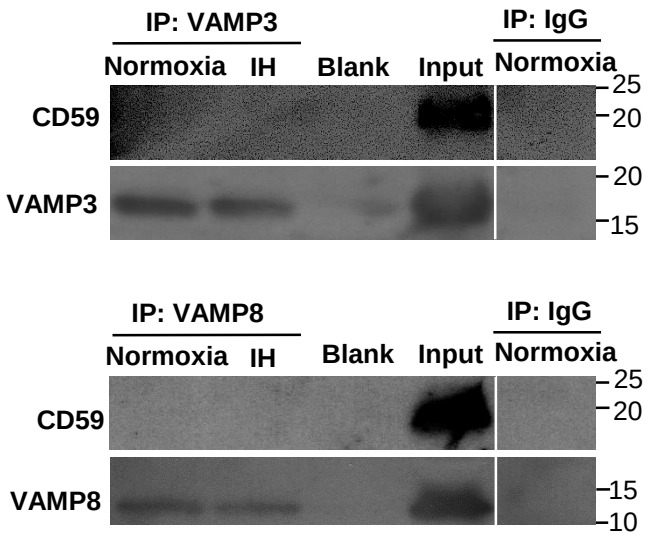
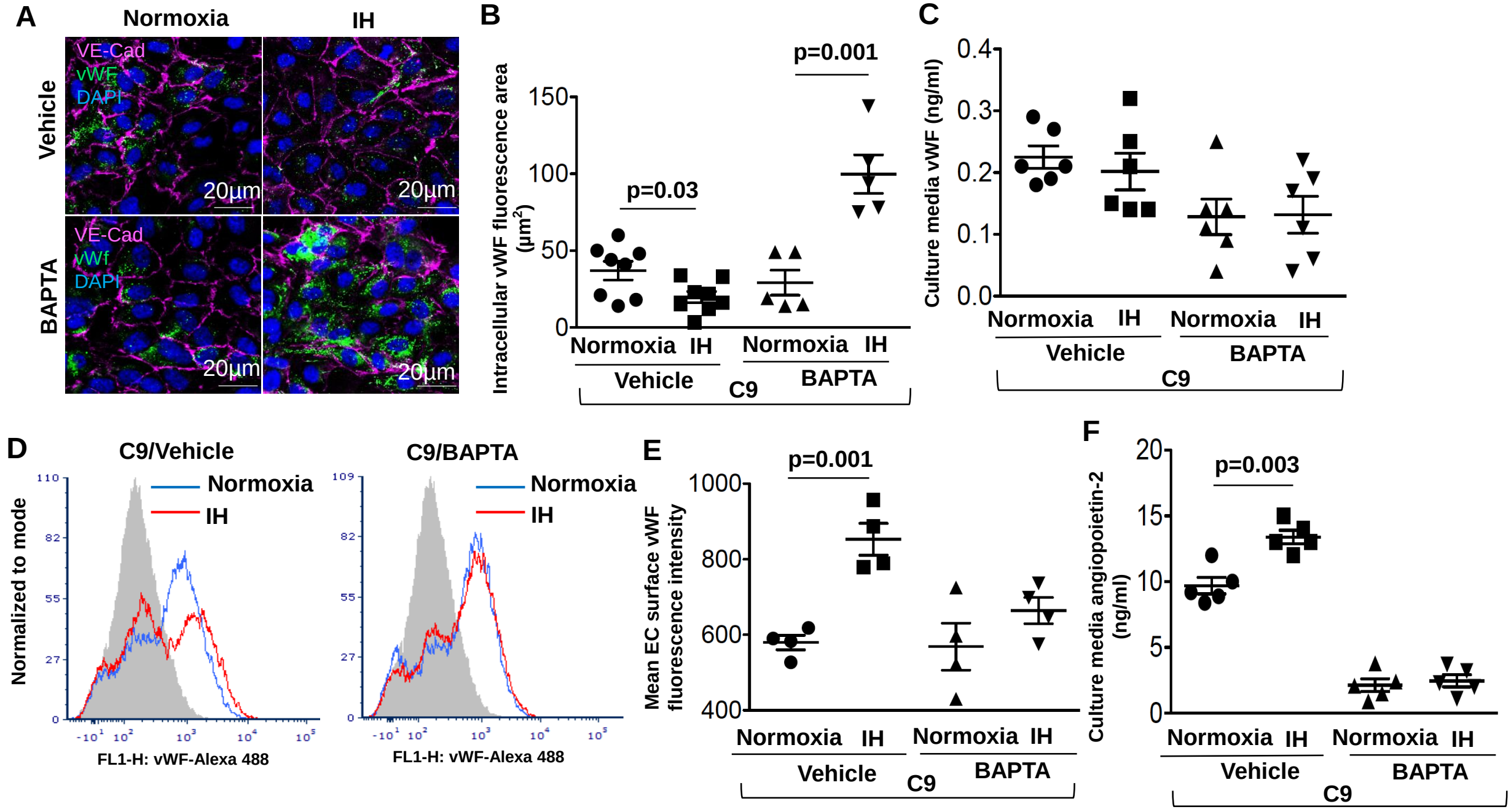

IP: VAMP3
IP: IgG
Normoxia
IH
Blank
Input
Normoxia
CD59
VAMP3
25
20
20
15
IP: VAMP8
IP: IgG
Normoxia
IH
Blank
Input
Normoxia
CD59
VAMP8
25
20
15
10

Figure S6


A
Normoxia
IH
Vehicle
BAPTA
VE-Cad
vWF
vWf
DAPI
20µm
B
Intracellular vWF fluorescence area (µm²)
p=0.001
p=0.03
Normoxia
IH
Vehicle
C9
BAPTA
C
Culture media vWF (ng/ml)
D
C9/Vehicle
C9/BAPTA
Normalized to mode
FL1-H: vWF-Alexa 488
E
Mean EC surface vWF fluorescence intensity
F
Culture media angiopoietin-2 (ng/ml)
p=0.003