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Vitamin D—A New Perspective in Treatment of Cerebral Vasospasm

BACKGROUND: Cerebral vasospasm (CVS) is a frequent complication after subarachnoid hemorrhage (SAH), with no sufficient therapy and a complex pathophysiology.
OBJECTIVE: To explore the vitamin D system as a potential treatment for CVS.
METHODS: 25-vitamin D3 levels tested between 2007 and 2015 and data of SAH patients admitted during the months with a peak vs nadir of VitD3 values were analyzed, retrospectively. We prospectively correlated VitD3 and vasospasm/outcome data in SAH patients admitted in 2017. An experimental mice SAH model and cell culture model were used to investigate the effect of 1,25-dihydroxyvitamin D3 (1,25-VitD3). Additionally, the mediators acting in the VitD mechanism were researched and detected.
RESULTS: Based on the retrospective analysis demonstrating an increased frequency of vasospasm in SAH patients during the low vitamin D period in winter, we started basic research experiments. Active 1,25-VitD3 hormone attenuated CVS, neurological deficit, and inflammation after intrathecal blood injection in mice. Deletion of the vitamin D receptor in the endothelium or in myeloid cells decreased the protective 1,25-VitD3 effect. Co-culture experiments of myeloid and endothelial cells with blood confirmed the anti-inflammatory 1,25-VitD3 effect but also revealed an induction of stroma-cell-derived factor 1α (SDF1α), vascular endothelial growth factor, and endothelial nitric oxide synthase by 1,25-VitD3. In mice, SDF1α mimicked the protective effect of 1,25-VitD3 against CVS. From bench to bedside, CVS severity was inversely correlated with vitamin D plasma level, prospectively. Patients with more severe CVS exhibited attenuated expression of SDF1α and 1,25-VitD3-responsive genes on circulating myeloid cells.
CONCLUSION: 1,25-VitD3 attenuates CVS after SAH by inducing SDF1α. However, VitD administration should be tested as optional treatment to prevent CVS.

KEY WORDS: Vitamin D, Subarachnoid hemorrhage, Cerebral vasospasm, Translational study

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Cerebral vasospasm (CVS) is a feared complication of subarachnoid hemorrhage (SAH) resulting in secondary stroke.[1] Current CVS-treatment strategies, ie, application of the calcium channel blocker nicardipine or the endothelin-1 antagonist clazosentan, failed to improve patient outcomes.[2]

The CVS pathomechanism involves inflammatory activation.[3] Because of the complex pathophysiology involving multiple pathways, it is not unexpected that simple vasodilator or anti-inflammatory therapy fail to substantially impact on the CVS severity.

1,25-dihydroxycholecalciferol (1,25-VitD3) improves vascular regeneration in response to local vascular-injury.[4] In addition, 1,25-VitD3 also has anti-inflammatory effects,[5] alters the myeloid cell differentiation, and has been suggested to induce protective vascular genes.[6] Therefore, we hypothesized that VitD may prevent the vascular processes leading to CVS after SAH.

ABBREVIATIONS: BA, basilar artery; **CI,** confidence interval; **CVS,** cerebral vasospasm; **DMSO,** dimethyl sulfoxide; **eNOS,** endothelial nitric oxide synthase; **HUVEC,** human umbilical vein endothelial cells; **mRS,** modified Rankin Scale; **OR,** odds ratio; **SAH,** subarachnoid hemorrhage; **RT-qPCR,** quantitative reverse transcription PCR; **SDF1α,** stroma-cell-derived factor 1α; **VEGF,** vascular endothelial growth factor; **VDR,** Vitamin D receptor

Supplemental digital content is available for this article at www.neurosurgery-online.com.

Accordingly, 1,25-VitD3 deficiency is linked to an increased risk of cardiovascular disease, stroke, and autoimmune disorders.[7] Patients requiring treatment for cerebral aneurysms have an increased VitD deficiency incidence,[8] and a high VitD deficiency prevalence has been noted among SAH patients.[9] Despite the absence of an association between VitD deficiency and outcomes in a first case-control-study including 33 patients,[9] further studies are needed to assess potential effects of VitD deficiency to reach an evidence-based conclusion.

Against this background, we determined the VitD impact on vascular function in a mouse SAH model. Translational studies in SAH patients were performed to demonstrate the clinical background and utility of our experimental findings. Trial registration can be found at http://www.clinicaltrials.gov with the unique identifier NCT03482843.

METHODS

Detailed method-section is reported in the online **Text**, **Supplemental Digital Content 1**.

RESULTS

Favorable Outcome in SAH Patients Admitted in Summer

25-VitD3 levels of registered patients during 2007 to 2015 (n = 95 036) were retrospectively analyzed. We stratified two groups, admissions in "summer" vs "winter" (highest vs lowest 25-VitD3 level), annually. 288 SAH patients were included (winter: n = 157; summer: n = 121). Patients with an advanced-brain-injury and patients lost for follow-up were excluded (n = 10). Concerning aneurysm rupture, there was a significantly higher rate of hemorrhage in the winter-group (56% vs 44% in summer; $P < .01$; odds ratio [OR] 1.7). This difference was associated with a significant higher rate of neurological deficits at admission indicated as Hunt & Hess grades ($P < .0001$; OR 7.9). In addition, SAH patients in summer had a statistically significant lower rate of early hydrocephalus ($P < .05$; OR 1.8), CVS ($P < .05$; OR 1.7) and followed delayed ischemic neurological deficits ($P < .01$; OR 2) and had a higher chance for favorable outcome (modified Rankin Scale [mRS]: 0-2)[10] ($P < .05$; OR 1.7) (Table 1A).

In multivariate analysis, seasonal SAH occurrence was significantly associated with the SAH prognosis as an independent factor. This finding was confirmed by ordinal regression with mRS as the dependent variable and seasonal SAH onset and further patient characteristics as independent variables (Table 1B).

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1,25-VitD3 Attenuates SAH-Induced Vascular Dysfunction and Neurological Deficits

We determined the VitD effect in a mouse SAH model. 12 h after blood application, the cerebral vessel diameter, determined ex Vivo, was reduced by approx. 1/3 (Figure 1A) and endothelial dysfunction was present as determined by endothelium-dependent, acetylcholine-induced relaxation of the isolated basilar artery (BA) (Figure 1B). These alterations were accompanied by a neuroscore increase (**Table**, **Supplemental Digital Content 2**) and a significant weight loss due to insufficient food/water intake (Figure 1C and 1D). The vessel diameter and endothelium-dependent relaxation in the SAH group receiving 1,25-VitD3 were significantly greater than in the control group. 1,25-VitD3 also prevented weight loss and attenuated the development of neurological deficits.

There was an association between CVS degrees and unfavorable outcome using 14-point score.[11] 1,25-VitD3-pretreated SAH mice had a significant lower risk for severe vasospasm and unfavorable outcome (Figure 1E).

1,25-VitD3 Limits SAH-Induced Inflammation and Induces Protective Genes

BA vascular gene expression (**Table Supplemental Digital Content 3**) was analyzed. The SAH model resulted in cytokine tumor necrosis factor α and interleukin-6 induction (Figure 1F). Although 1,25-VitD3 largely prevented the SAH-induced inflammation, it did not prevent vascular macrophage accumulation in response to SAH as detected by the abundance of the macrophage-marker EMR1 (Figure 1F). It was therefore interesting to note that only the combination of SAH and 1,25-VitD3 resulted in an induction of vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), and the stroma-cell-derived factor1α (SDF1α) (Figure 1G). Importantly, neither the SAH model nor 1,25-VitD3 alone had a strong effect on these genes on its own, and their massive induction only occurred if 1,25-VitD3 was applied to the SAH group. This suggests that 1,25-VitD3 and SAH stimulate distinct signaling pathways that add up to the eventual induction of protective genes. Furthermore, 1,25-VitD3 pre-treatment induced VitD-responsive genes (Figure 1G).

The Protective Effect of 1,25-VitD3 Occurs in Tissue-Resident Vascular Cells

Organ cultures of mouse BA co-incubated with blood were performed as this model excludes cellular recruitment. The gene-expression pattern observed after 48 h of organ culture largely resembled those of the in Vivo situation (**Figure**, **Supplemental Digital Content 4A-C**).

TABLE 1A. Seasonal Difference in SAH Patients Admitted between 2007 and 2015

2007-2015	Summer cohort	Winter cohort	*P**	OR*
Number of patients (N = 278)	121 (44%)	157 (56%)	**<.01**	**1.7 (1.2-2.4)**
Female	82 (29%)	109 (39%)	NS*	–
Hunt & Hess ≤ III	103 (37%)	66 (24%)	**<.0001**	**7.9 (4.4-13.9)**
Fisher 3 blood pattern	68 (24%)	108 (39%)	**<.05**	**1.7 (1-2.8)**
Early hydrocephalus	70 (25%)	112 (40%)	**<.05**	**1.8 (1.1-3)**
CVS	36 (13%)	66 (24%)	**<.05**	**1.7 (1.026-2.853)**
H&H I-III	26	24	<.001	4.5 (1.9-11.5)
H&H > III	10	42	<.001	0.2 (0.1-0.5)
Fisher 1-2 blood pattern	9	31	<.05	0.4 (0.2-0.9)
Fisher 3-4 blood pattern	27	35	<.05	2.7 (1.1-6.1)
Cerebral infarction	46 (17%)	59 (21%)	NS	–
Delayed ischemic neurological deficits	68 (25%)	113 (41%)	**<.01**	**2 (1.195-3.291)**
Shunt	27 (10%)	43 (16%)	NS	–
Favorable outcome (mRS* 0-2) (6 mo after SAH)	98 (35%)	59 (21%)	**<.05**	**1.7 (1.082-2.845)**

Data are shown in n (%); *$P < .05$ is significant. Odd ratio (OR) data with 95% CI.

TABLE 1B. Clinical Outcome in Association With Patient Characteristics in SAH Patients Admitted between 2007 and 2015

2007-2015 patients characteristics	Favorable outcome	Unfavorable outcome	Multivariate analysis *P* value	OR (95% CI)	Ordinal regression Prob > Chi^2	Chi^2
Number of patients (n = 278)	157 (56%)	121 (44%)	–	–	–	–
Age < 50 yr	67 (55%)	54 (45%)	.72	.90 (.52-1.57)	0.72	0.13
Female	109 (57%)	82 (43%)	.56	.84 (.47-1.51)	0.55	0.34
Sah onset in summer	**98 (81%)**	**23 (19%)**	***<.0001**	**6.45 (3.45-12.05)**	***<0.0001**	**34.14**
Sah onset in winter	**59 (38%)**	**98 (62%)**	***<.0001**	**.16 (.08-0.29)**	***<0.0001**	**34.14**
Hunt & Hess ≤ III	112 (66%)	57 (34%)	.45	.70 (.39-1.26)	0.24	1.39
Fisher 3 blood pattern	90 (51%)	86 (49%)	.35	.76 (.43-1.35)	0.35	0.88
Early hydrocephalus	62 (34%)	120 (66%)	.37	.76 (.42-1.38)	0.37	0.80
Hypertension	35 (56%)	28 (44%)	.44	.78 (.41-1.48)	0.44	0.59
Anticoagulation	14 (67%)	7 (33%)	.45	.66 (.23-1.93)	0.45	0.58
Nicotine abuse	46 (57%)	35 (43%)	.92	.97 (.54-1.75)	0.92	0.01
Positive family history	13 (57%)	10 (43%)	.48	.7 (.26-1.87)	0.48	0.51
Clipping	84 (59%)	58 (41%)	.29	.37 (.06-2.29)	0.29	1.14
Coiling	78 (55%)	65 (45%)	.31	.39 (.06-2.4)	0.31	1.04
Minority Group (Asia, Middle East, Africa, and South America)	18 (58%)	13 (42%)	.50	.74 (.32-1.75)	0.50	0.46

Favorable outcome 6 mo after SAH onset: mRS ≤ 2 points; Unfavorable outcome: mRS > 2 points. SAH onset in summer: patients admitted during 2 mo with the highest VitD levels per year. SAH onset in winter: patients admitted during 2 mo with the lowest VitD levels per year. Data are shown in n (%); Multivariate analysis: *$P < .05$ is significant. Odd ratio (OR) data with 95% CI. Ordinal regression analysis: *Prob. > Chi Square (Prob > Chi^2) <0.05, Chi Square (Chi^2).

These findings demonstrate that in an ex Vivo SAH model, tissue-resident myeloid cells are sufficient to induce the protective 1,25-VitD3 effect.

Tissue-Resident LysM-Positive Cells and Endothelial Cells Both Mediate the 1,25-VitD3 Effect

In order to differentiate the 1,25-VitD3 effect on the endothelium and on tissue-resident macrophages, the vitamin D receptor (VDR) receptor was selectively deleted in these cells using the cre-lox system. In both mouse lines, the protective 1,25-VitD3 effects were basically lost, and the VDR target gene induction was attenuated (Figure 2). Different to these observations, SDF1α induction was lost in response to myeloid-specific VDR deletion, whereas its expression was even potentiated in response to endothelial VDR ablation. SDF1α might be required for 1,25-VitD3-dependent macrophage–endothelial crosstalk.

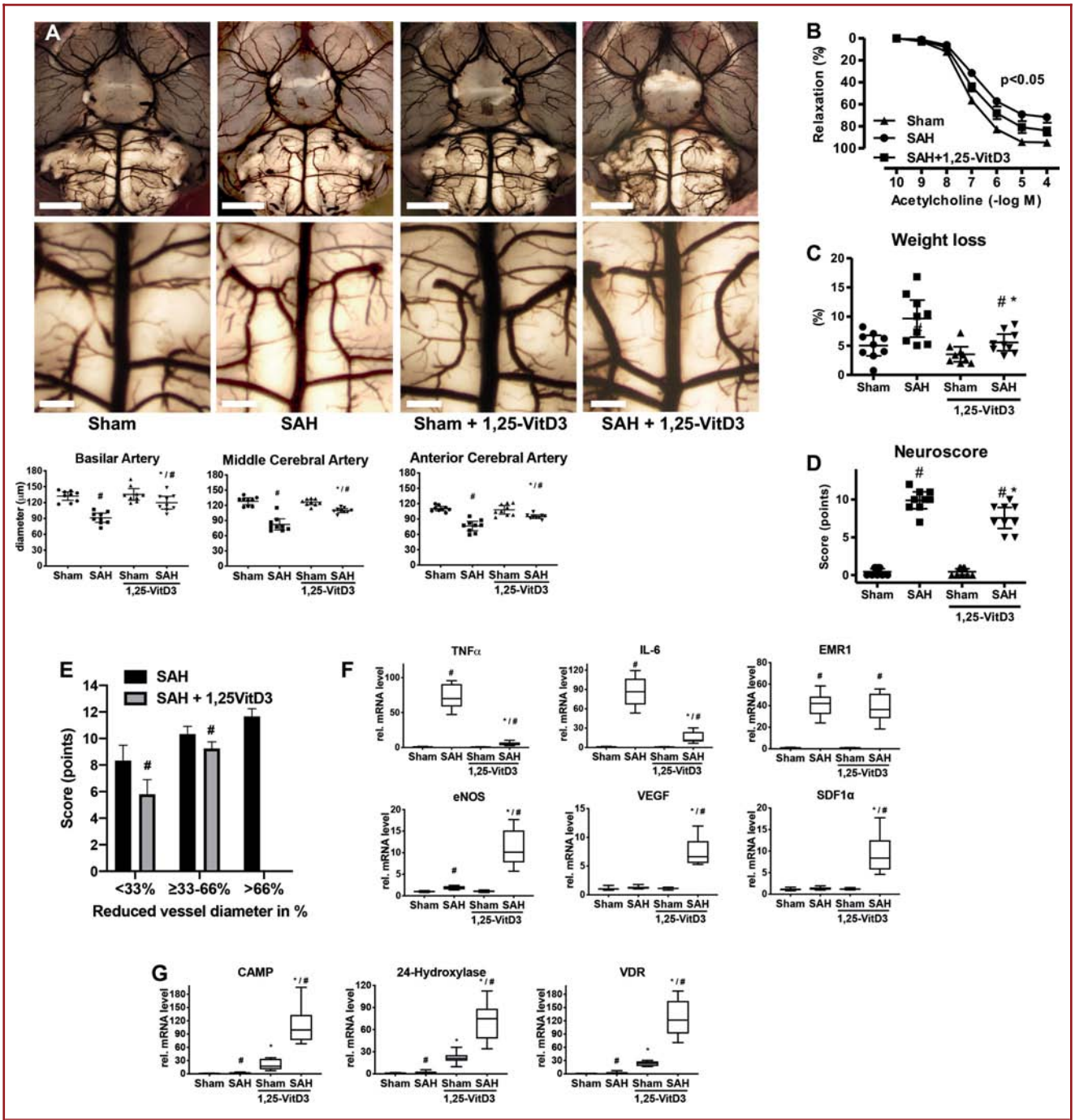

FIGURE 1. *1,25-VitD3 attenuates vascular dysfunction in a murine SAH model.* **A**, *Representative ink images of the cerebral vasculature and BA in magnification and statistics of vessel diameter of mice 12 h after intrathecal injection of blood (SAH, 60 µl) or saline (sham) with and without 1,25-VitD3 pretreatment (100 ng/kg/d i.p.; 5 d). n = 9. ANOVA, Fisher test, #P < .05 sham vs SAH. *P < .05 with vs without 1,25-VitD3. Mean with 95% (CI). Scale bars are 1000 and 200 µmeters, respectively.* **B**, *Endothelium-dependent relaxation of the isolated BA harvested from the animal groups indicated (n = 5). ANOVA for repeated measurements. *P < .05. Mean with 95% CI.* **C** *and* **D**, *Relative weight loss, ANOVA, Fisher test* **C**, *and neuroscore, ANOVA Mann-Whitney test* **D**, *n = 9, #P < .05 sham vs SAH. *P < .05 with vs without 1,25-VitD3. Shown are mean +/− 5/95% CI.* **E**, *Correlation between different CVS degrees defined as reduced diameter of BA in percent compared to the mean BA diameter in mice (<33%, ≥33%-66%, and > 66%) and worse outcome using the 14-point score by Chen et al (2001).*[11] *n = 9. Multiple t-test. #P < .05 SAH with vs without 1,25-VitD3. Mean with 95% CI.* **F** *and* **G**, *quantitative reverse transcription PCR (RT-q PCR) polymerase chain reaction (PCR) of the genes indicated from the BA of mice 12 h after intrathecal injection of blood (SAH, 60 µl) or saline (sham) with and without Control 1,25-VitD3 pretreatment (100 ng/kg/d i.p.; 5 d). n = 9. ANOVA, Fisher test, #P < .05 sham vs SAH. *P < .05 with vs without 1,25-VitD3. Mean with 95% CI.*

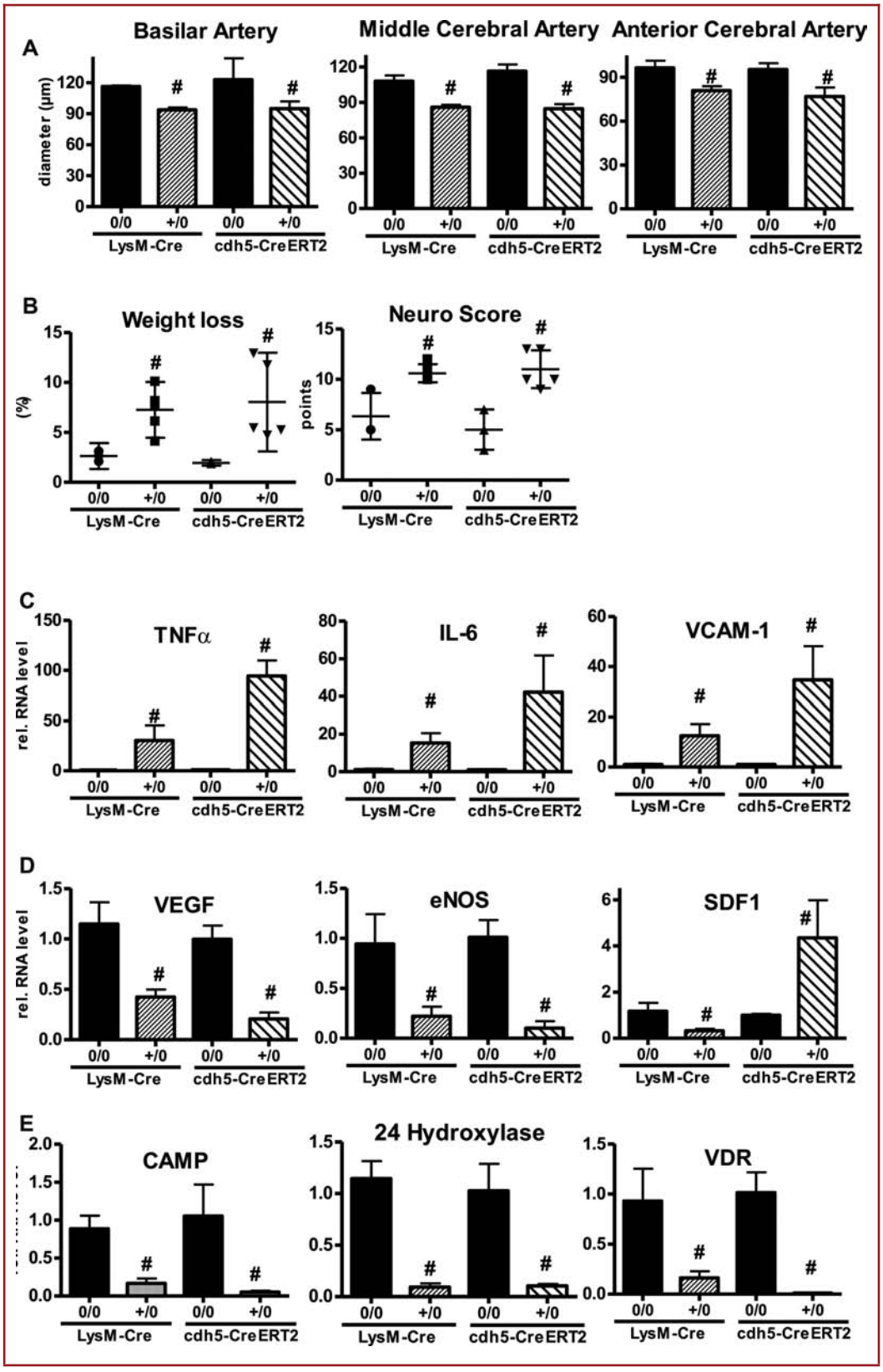
A
Basilar Artery
Middle Cerebral Artery
Anterior Cerebral Artery
diameter (μm)
B
Weight loss
(%)
Neuro Score
points
C
TNFα
IL-6
VCAM-1
rel. RNA level
D
VEGF
eNOS
SDF1
rel. RNA level
E
CAMP
24 Hydroxylase
VDR
0/0
+/0
LysM-Cre
cdh5-CreERT2

FIGURE 2. *The protective effect of 1,25-VitD3 requires the vitamin D receptor in LysM-positive cells and in the endothelium.* **A**-**E**, *Statistics of vessel diameter* **A**, *weight loss, and neuroscores* **B** *of the VDR flox-flox mouse lines indicated 12 h after intrathecal injection of blood (SAH, 60 μl) and after 1,25-VitD3 pre-treatment (100 ng/kg/d i.p.; 5 d). n = 3 (cre0/0) and n = 5 (cre+/0). ANOVA, #P < .05 cre0/0 vs cre0/+, shown are mean +/− 5/95% CI.* **C**-**E**, *qRT-PRT of the genes indicated from the BA of the mice lines indicated 12 h after intrathecal injection of blood (SAH, 60 μl) with 1,25-VitD3 pretreatment (100 ng/kg/d i.p.; 5 d). ANOVA, Fisher test, #P < .05 cre0/0 vs cre0/+, shown are mean +/− 5/95% CI.*

Macrophages Mediate the Induction of Protective Genes in the Endothelium in Response to 1,25-VitD3

To identify such a potential crosstalk between macrophages and the endothelium, macrophages differentiated from human blood and human umbilical vein endothelial cells (HUVEC) were studied separately or in co-culture in the presence or absence of blood and 1,25-VitD3. Blood alone had no effect on the expression of inflammatory or protective genes in HUVEC (Figure 3). In HUVEC monoculture, 1,25-VitD3-induced VDR-response genes did not affect the expression of protective or inflammatory genes. Blood induced an inflammatory reaction in the macrophage monoculture that was attenuated by 1,25-VitD3. 1,25-VitD3 induced VDR target genes, but expression of VEGF and eNOS remained unaffected (Figure 3). Interestingly, whereas blood slightly induced SDF1α in macrophages, this response was potentiated by 1,25-VitD3, which by itself had little effect of basal SDF1α expression.

During co-culture, macrophage gene expression was similar to that in monoculture, only more pronounced (Figure 3). Interestingly, macrophage presence resulted in an inflammatory reaction of HUVEC in response to blood that was greatly attenuated by 1,25-VitD3 (Figure 3). VEGF and eNOS were greatly induced by the combination of blood and 1,25-VitD3, whereas 1,25-VitD3 alone had little effect at all. These data demonstrate that tissue-resident macrophages instruct endothelial cells to respond to blood in a pro-inflammatory manner. 1,25-VitD3 blocks the inflammatory response and raises a macrophage-dependent, protective SDF1α-mediated response in the endothelium.

SDF1α Mediates the Protective Effects of 1,25-VitD3 during SAH In Vivo

Subarachnoidal SDF1α co-administration with blood had a similar protective effect as 1,25-VitD3 (Figure 4). The vessel diameter increased, neurological deficits and weight loss were attenuated, and the inflammatory response to SAH was attenuated (Figure 4A-4C). SDF1α also induced the protective genes VEGF and eNOS, and this effect was even greater than in response to 1,25-VitD3 (Figure 4D). This suggests that the 1,25-VitD3 effects are mediated through SDF1α release from tissue-resident macrophages. To test this, mice were treated with the SDF1α receptor blocker AMD3100 at the time of blood application. AMD3100 blocked the protective effects of 1,25-VitD3 and prevented the induction of VEGF and eNOS (Figure 4D). These findings suggest that blood raises an inflammatory response that is mediated by macrophages and results in CVS. 1,25-VitD3 program macrophages towards attenuated inflammation and SDF1α production. SDF1α subsequently reaches the endothelium and elicits a protective response that is further amplified by permissive 1,25-VitD3 effects on protective endothelial genes.

Favorable Outcome in SAH Patients with Higher Vitamin D Level

A total of 23 SAH patients consented to the prospective study. We determined 25-VitD3 plasma level, CVS severity, and gene expression in myeloid cells at different time points; no patient was lost to follow-up. High 25-VitD3 level was associated with significant lower CVS rate and higher chance for favorable outcome ($P < .05$; OR 18) (Table 2). In addition, we performed a multiple regression analysis including mRS data to describe the clinical outcome 6 mo post-SAH in association with VitD3 levels and the existence of hypertension, a disease with seasonal fluctuations. We observed that low VitD3 ($P < .0001$) but not hypertension ($P = .55$) is an independent factor influencing the SAH prognosis. 25-VitD3 plasma levels were inversely correlated with the CVS severity ($r = -0.63$; $P < .001$) (Figure 5A). VitD3 level also showed a significant inverse correlation with the clinical outcome scores (Spearman r correlation: $P < .0001$; $r = -0.8$) (Figure 5B). As compared to the values on time of admission, the expression of VitD-responsive genes (CAMP; VDR) was significantly higher during the time of CVS development and subsequently decreased again. Also, SDF1α was induced at CVS time (Figure 5C-5E). Subsequently, we compared the expression of VitD-responsive genes between the groups with no/mild vs moderate/severe CVS. Importantly, the induction of VitD-dependent genes was basically restricted to the group with no/mild CVS, whereas the group with moderate/severe CVS exhibited no induction of VitD-responsive genes (Figure 5F-5H). This difference was also observed for SDF1α, which we identified as the beneficial mediator of the VitD action in our animal experiments. Thus, a functional deficiency in the VitD system appears to contribute to CVS in patients.

DISCUSSION

Low 25-VitD3 levels in SAH patients are associated with CVS and unfavorable outcome. 1,25-VitD3 potently inhibits the negative vascular effects in a murine SAH model. Mechanistically, the combination of blood and 1,25-VitD3 resulted in SDF1α production by tissue-resident myeloid cells. SDF1α subsequently promoted the induction of protective genes in the endothelium.

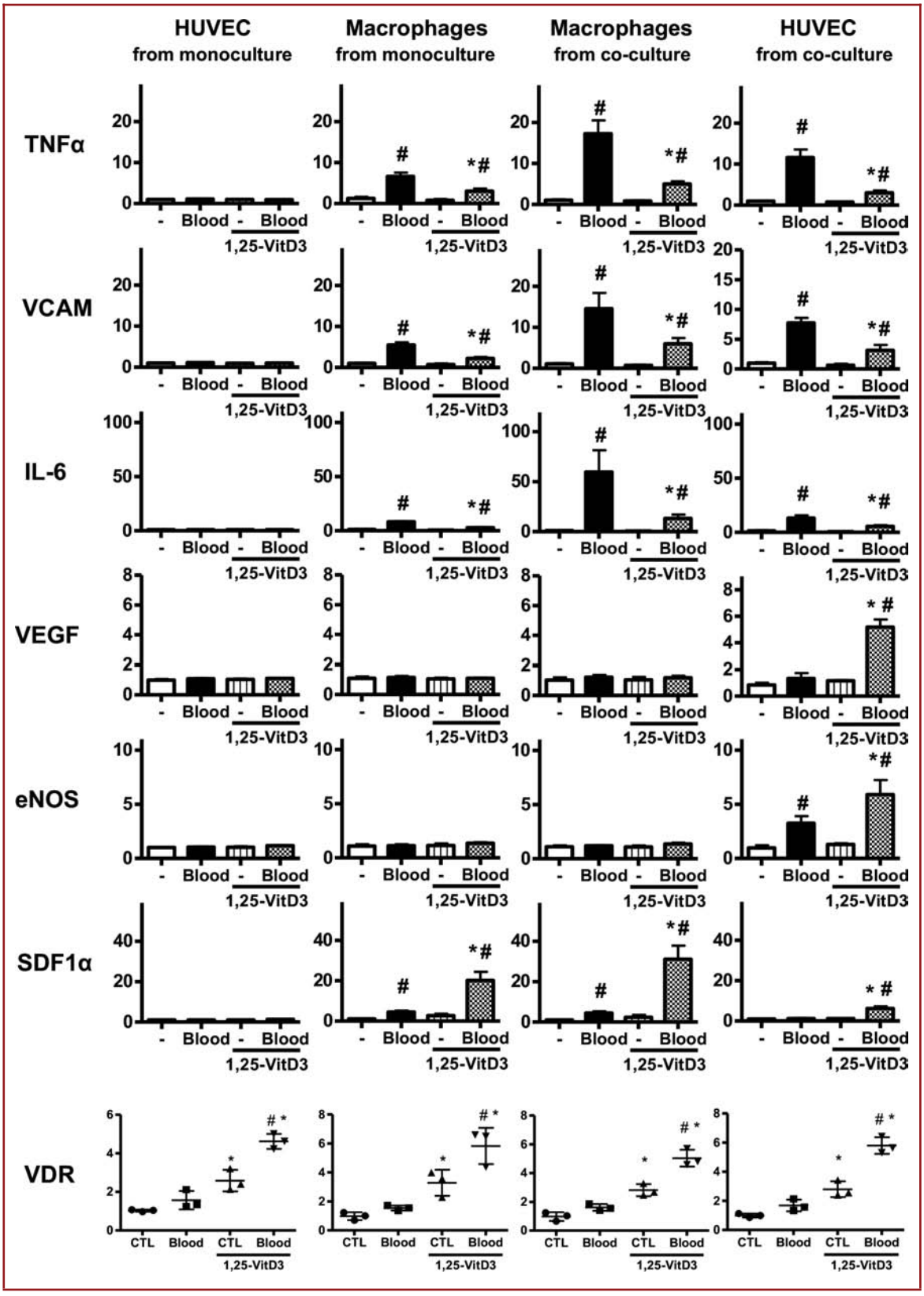
HUVEC
from monoculture
Macrophages
from monoculture
Macrophages
from co-culture
HUVEC
from co-culture
TNFα
VCAM
IL-6
VEGF
eNOS
SDF1α
VDR
- Blood - Blood
1,25-VitD3
CTL Blood CTL Blood
1,25-VitD3

FIGURE 3. *Macrophages induce protective genes in the endothelium in response to 1,25-VitD3. Relative expression changes as determined by RT-qPCR of the genes indicated from the cells denoted. Cells were kept in mono- or co-culture for 48 h in the presence or absence of blood (10% blood in endothelial cell basal medium (EBM)) and presence or absence of 1,25VitD pretreatment (100 nmol/L in EBM; Control: Dimethyl sulfoxide (DMSO)), n = 3. ANOVA, Fisher test, #P < ,05 with vs without blood. *P < ,05 with vs without 1,25-VitD3. Shown are Mean +/− 5/95% CI.*

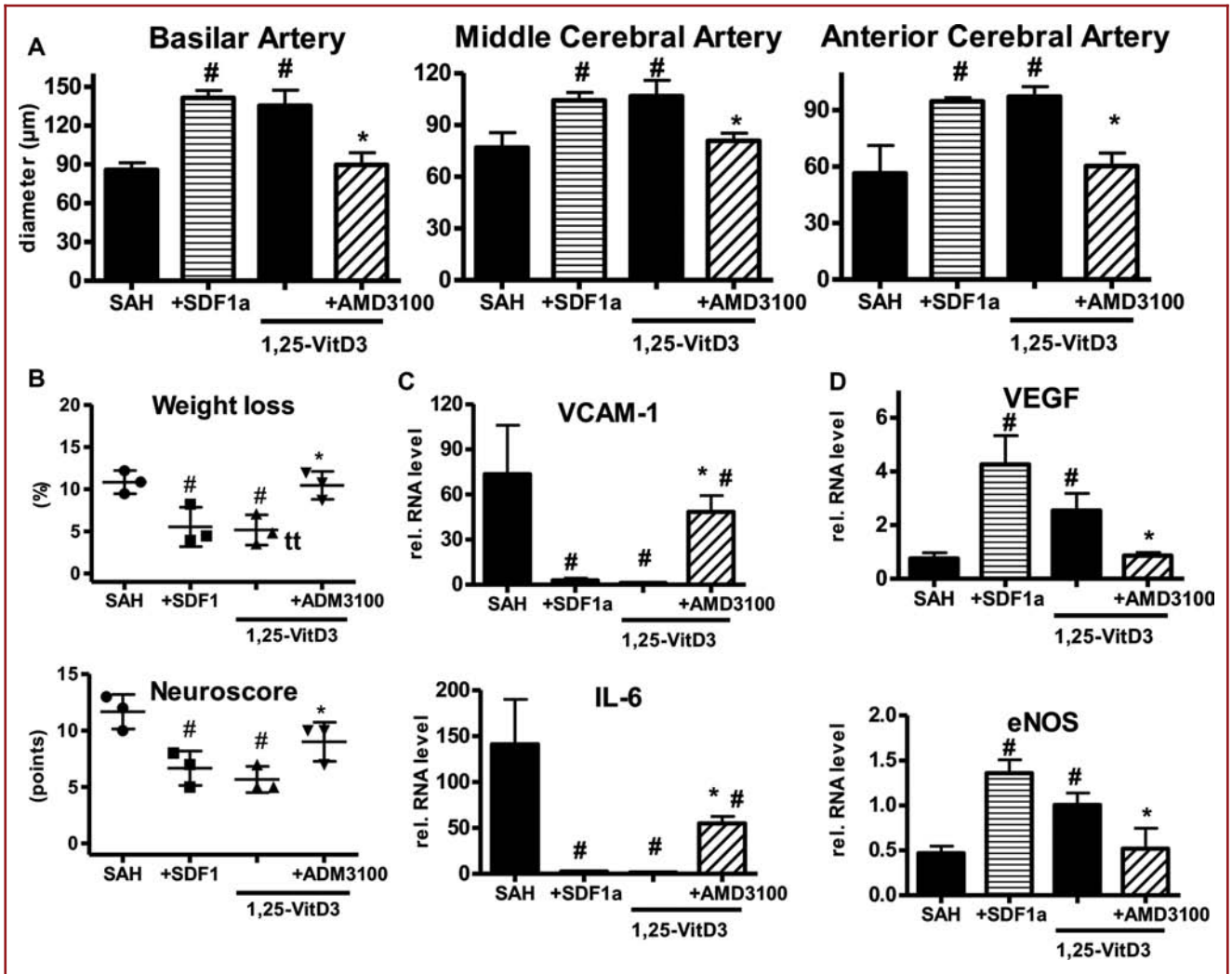

FIGURE 4. *SDF1α prevents SAH-induced vascular dysfunction and mediates the effects of vitamin D.* **A**, *Statistics of vessel diameter of mice 12 h after intrathecal injection of blood (SAH, 60 µl) with and without 1,25-VitD3 pretreatment (100 ng/kg/d i.p.; 5 d). In subgroups, the CXCR4 receptor antagonist AMD3100 (5 mg/kg, i.p.) or SDF1α (250 ng intrathecal) were administered at time of blood injection (n = 3). ANOVA, Fisher test,* **B**, *Relative weight loss, ANOVA, Fisher Test, and neuroscore, ANOVA Mann-Whitney test.* **C** *and* **D**, *RT-qPCR of the genes indicated from the BA of mice 12 h after intrathecal injection. ANOVA, Fisher test. #P < .05 SAH vs SAH + 1,25-VitD3 or SAH + SDF1α. *P < .05 SAH + 1,25-VitD3 vs SAH + 1,25-VitD3 + AMD3100. Shown are mean +/− 95% CI.*

VitD-response genes, including SDF1α, were induced in circulating myeloid cells during the clinical course of SAH and peaked at times of CVS. Patients with severe CVS did not exhibit this response.

SDF1α Mediator of the Protective Effects of Vitamin D

SDF1α and its receptor CXCR4 are known for their function in homing of progenitor cells.[12] CXCR4 dysfunction in cardiovascular disease context results in attenuated homing of angiogenic myeloid cells and reduced vascular repair.[13] CXCR4 is expressed on endothelial and smooth muscle cells. Its function is largely atheroprotective, but the maintenance of vascular barrier function is mediated by SDF1α.[14] High SDF1α plasma levels have been associated with hemorrhagic severity and poor outcome after SAH.[15] The cytokine is a HIF1α-dependent gene and inflammation promotes HIF1α stabilization.[16] Thus, high

TABLE 2. Patient Characteristics at Admission, Clinical Complications, and Clinical Outcome in SAH Patients in Dependence on 25-VitD3 Level in 2017

2017 patients characteristics	25-VitD3 level < 25 ng/ml	25-VitD3 Level 25-70 ng/ml	Univariate analysis *P* value	OR (95% CI)
Number of Patients (N = 23)	16 (43%)	7 (57%)	–	–
Age < 50 yr	2 (50%)	2 (50%)	.56	0.36 (0.05-2.89)
Female	5 (56%)	4 (44%)	.36	0.34 (0.07-1.91)
Hunt & Hess ≤ III	9 (64%)	5 (36%)	.66	0.51 (0.09-2.87)
Fisher 3 blood pattern	6 (67%)	5 (33%)	.19	0.24 (0.04-1.43)
Hypertension	3 (60%)	2 (40%)	.62	0.58 (0.1-4.1)
Anticoagulation	2 (50%)	2 (50%)	.56	0.36 (0.05-2.88)
Nicotine abuse	5 (63%)	3 (37%)	.66	0.61 (0.1-3.2)
Positive family history	1 (50%)	1 (50%)	.53	0.4 (0.02-8.78)
Clipping	8 (80%)	2 (20%)	.4	2.5 (0.44-14.9)
Coiling	8 (62%)	5 (38%)	.4	0.4 (0.07-2.27)
Minority group (Asia, Middle East, Africa, and South America)	2 (67%)	1 (33%)	>.9999	0.86 (0.09-14.3)
Severe CVS	**12 (92%)**	**1 (8%)**	***<.05**	**0.56 (0.005-0.61)**
Cerebral infarction	8 (80%)	2 (20%)	.4	2.5 (0.44-14.9)
Delayed ischemic Neurological deficits	7 (88%)	1 (12%)	.35	4.7 (0.6-60.19
Favorable outcome 6 mo after SAH (mRS ≤ 2)	**4 (40%)**	**6 (60%)**	***<.05**	**18 (1.63-198.5)**

Favorable outcome 6 mo after SAH onset: mRS ≤ 2 points; unfavorable outcome: mRS > 2 points. Data are shown in n (%); *$P < .05$ is significant. Odd ratio (OR) data with 95% CI.

SDF1α plasma level reflects severe hypoxia and injury. Indeed, in a previous study about VitD-facilitated vascular repair, we demonstrated that an interaction of the VitD system with HIF1α mediates SDF1α induction.[4] Interestingly, this also implies a rather local 1,25-VitD3 action precisely at sites of injury. Such a guided SDF1α induction is attractive, as it will limit side effects and still allows for maximal local therapeutic effects. Indeed, coating of coils with SDF1α has been successfully used to promote cerebral aneurysm organization and healing.[17]

What is the Target Cell of 1,25-VitD3 After SAH?

The classic 1,25-VitD3 signaling depends on the nuclear VitD receptor,[18] and thus, sufficient VDR expression is a prerequisite for 1,25-VitD3 signaling. Myeloid cells express significant VDR level, whereas expression in vascular cells is rather low.[18] The central nervous system and its meningeal coverings accommodate a diverse myeloid compartment.[19] Perivascular macrophages preserve the health of endothelial cells, promote capillary stability, regulate vascular constriction, and maintain blood-brain barrier.[20] They are well situated to monitor CNS and peripheral homeostasis as well as changes in the subarachnoid space after a hemorrhage. Thus, it was an unexpected finding that 1,25-VitD3 protective effects were not only lost upon VDR deletion in myeloid cells but also after deletion in endothelial cells. These findings are in contrast to our previous work on vascular regeneration, in which the 1,25-VitD3 action was mediated by myeloid cells.[4] Potentially, VDR expression in the brain differs from that in the periphery, or VDR is induced by inflammatory activation. Indeed, VDR is induced by 1,25-VitD3 itself,[16] and even the clinical data of the present study demonstrate that inflammation induces the receptor and thus sensitizes to VitD.

Vitamin D Target Gene Induction After SAH

VitD signaling in myeloid cells is potentiated by inflammation through pathways involving Nuclear factor kappa-light-chain-enhancer of activated B cells and c-Jun N-terminal kinase.[18] It could therefore be expected that VDR-response genes were induced in the clinical course after SAH during intensive care unit stay. It was, however, unexpected that patients with severe CVS exhibited the lowest VDR target gene induction. As SDF1α is among these genes, we interpret this finding as further evidence for an important causal protective SDF1α role post-SAH. VitD signaling is complex and controlled by numerous enzymes for all of which polymorphisms have been reported.[18] Moreover, the intrinsic VitD processing and signaling system of myeloid cells differs from that of epithelial cells. Larger studies therefore will be needed to dissect the genetic details underlying the differential response observed in the present study.

Vitamin D—-How Strong is the Evidence?

Although there is great interest in VitD, it is fair to state that the clinical data supporting therapeutic VitD application in the cardiovascular system are, at best, weak.[7] A significant number of small or poorly controlled studies supports VitD supplementation for a number of disease conditions, but prospective, well-controlled larger trials were largely negative.[21] This is surprising given the convincing mechanistic data for protective and

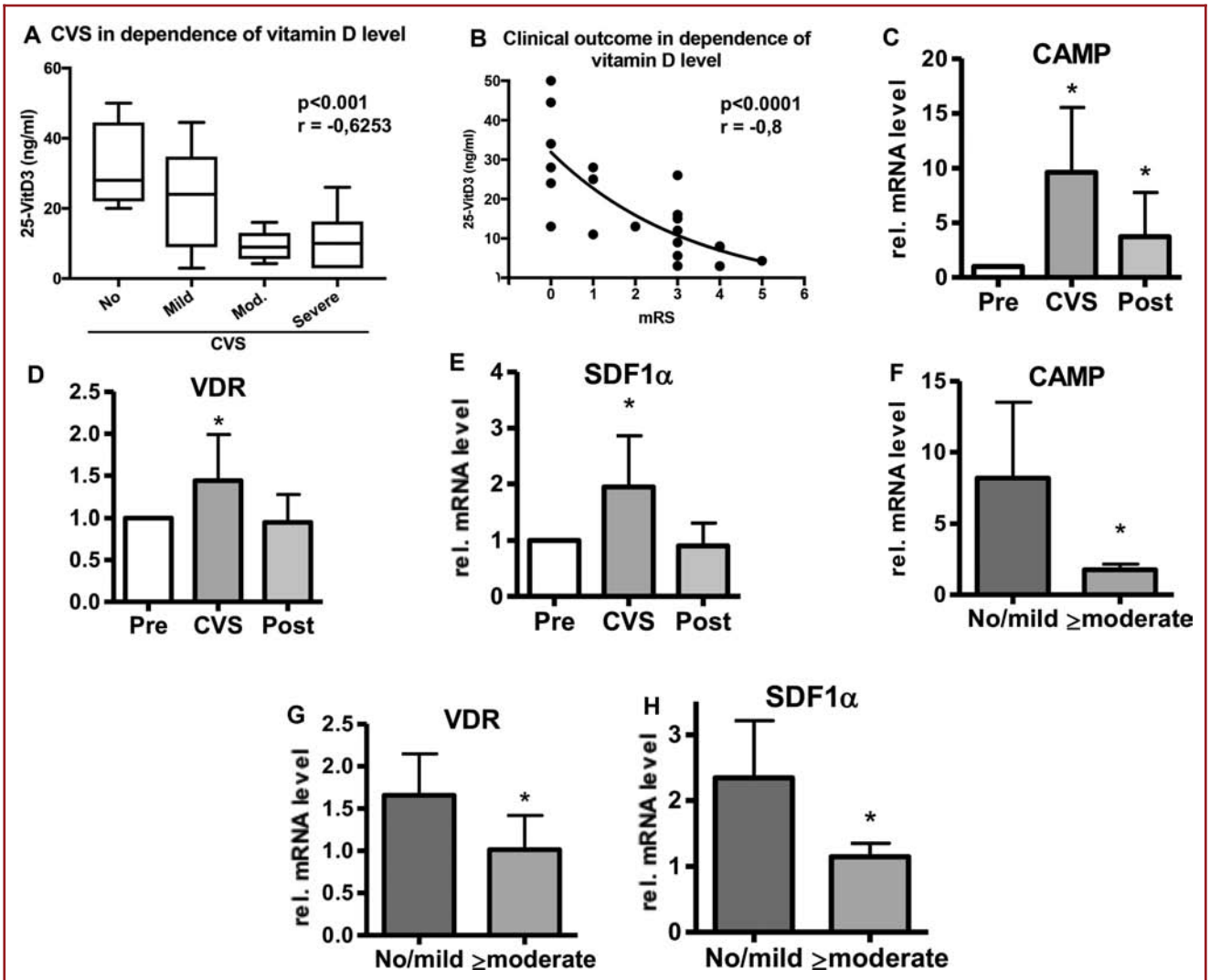

FIGURE 5. *Prospective study cohort: vitamin D is associated with favorable outcome in SAH patients.* **A**, *Spearman correlation of plasma vitamin D level on time of admission and degree of CVS. Patients (n = 23) were divided into 4 groups: no CVS (0% decrease of vessel diameter) (n = 5), mild CVS (<33% decrease of vessel diameter) (n = 5), moderate CVS (33-66% decrease of vessel diameter) (n = 7), and severe CVS (>66% decrease of vessel diameter) (n = 6).* **B**, *Spearman correlation of plasma vitamin D level on time of admission and clinical outcome level described as mRS 0 to 6 in 23 patients. P < .0001 is significant.* **C-H**, *RT-qPCR of the genes indicated from CD14-positive peripheral blood mononuclear cells isolated before (pre-CVS) during (CVS) and after (post) the CVS period.* **C-E**, *Pooled data of all patients. n = 16, ANOVA, *P < .05. Mean with 95% CI.* **F-H**, *Comparison of gene expression at time of CVS between patients with mild or no CVS (n = 8) to those with moderate and severe CVS (n = 8). ANOVA, *P < .05. Mean with 95% CI.*

anti-inflammatory VitD functions. VitD failure in larger trials might therefore be a consequence of an unselected patient inclusion. VitD status, skin type, age, and gender all impact on the response to supplementation.[18]

Vasospasm is a fairly late event, often occurring about a week after SAH. With so much time between ictus and onset of symptoms, it is hoped that treatments can be implemented in this window. Several studies suggest that inflammation after SAH begins early. VEGF and mitogen activation protein kinase are up-regulated early after experimental SAH.[22] Both factors are associated with innate inflammatory responses. Likewise, early, elevated levels of matrix metalloproteinase-9 and VEGF were associated with vasospasm in SAH patients.[23] Interestingly, the inhibition of lymphocyte function-associated antigen-1 within 3 h of the hemorrhage prevents vasospasm of isolated femoral arteries, suggesting that the inflammation process occurs very early in the course of SAH.[24] However, neutrophils are elevated in the first 3 d post-SAH; days before the onset of clinical symptoms or evidence of vasospasm.[25] In a mouse model of vasospasm, depletion of myeloid cells prior

to SAH prevents vascular spasm and development of behavioral deficits.[26]

The clinical data of the present study suggest a beneficial VitD effect in two independent clinical studies and link higher VitD levels to a reduced incidence and CVS severity and to favorable outcome (Tables 1A and 1B and 2). Our encouraging findings may therefore justify further clinical testing.

Limitations of the Study

Retrospective study has typical limitations like failure to record important parameters (individual VitD levels) and difficulties to define intervention groups. Thus, it was important that our prospective study supports the results of the retrospective analysis. All SAH mouse models have limitations,[27] and the model used here is characterized by early and short-lasting CVS. Thus, the findings cannot be directly transferred to humans, which are characterized by late-onset CVS. At this point, the pathomechanism affected by VitD in our experimental study should be defined as a correspondence to the term "early brain injury," demonstrating the clinical relevance of the short-term vascular spasm in our mouse model. Nevertheless, comparing CVS in SAH patients with the short-lasting vessel spasm followed by relevant clinical symptoms in mice should be mentioned as a main limitation of this study. But we used mice to enable a tissue-specific VDR deletion for detecting the mechanism of VitD effect. Before clinical studies with VitD in humans are performed, models in larger animals should be tested with respect to the VitD effectiveness. The rapid CVS onset in our animal-model also forced us to pretreat the mice with 1,25-VitD3. The compound acts on gene expression and thus administration at time of the SAH model was unlikely to be effective. Although this may limit the treatment options for VitD substitution, the situation in humans is different because of the delayed CVS occurrence. Thus, VitD application in humans after the SAH onset could be promisingly. In addition to the limitations according experimental part, we have to add that the neuroscore used here is observe dependent and prone to bias.

In humans, SAH is more common in females than in males. It is therefore a limitation that we focused on male mice to exclude estrus-cycle effects in order to reduce variability within the animal experiments.

Finally, because of small number of patients in the prospective study part, a larger and more sophisticated study will be needed to obtain definite results, optimally in a double-blinded, randomized, controlled trial.

CONCLUSION

Our clinical data suggest that VitD deficiency favors an unfavorable outcome post-SAH. 1,25-Vitamin D supplementation attenuated spasm development in a mouse model, which was mediated by an SDF1α induction. SDF1α mediated the protection in response to VitD in mice and patients with severe CVS failed to induce SDF1α in myeloid cells. Induction of VitD-responsive genes in myeloid cells could be valid as biomarker to predict the outcome after SAH. Collectively, VitD and potentially SDF1α should be clinically tested to prevent the CVS occurrence and improve outcome in SAH patients.

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Disclosures

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Supplemental Digital Content 1. Text. Material, methods, and statistical analysis.
Supplemental Digital Content 2. Table. 14 points neurological deficit score.
Supplemental Digital Content 3. Table. Primers.
Supplemental Digital Content 4. Figure. Funnel plot: blood and 1,25-VitD3 raise a similar gene-expression response in organ experiments as in Vivo. A-C, QRT-PRT of the genes indicated from the BA of mice kept in organ culture for 48 h in the presence or absence of blood (10% autologous blood in endothelial cell basal medium (EBM) and presence or absence of 1,25-VitD pre-treatment (100 nmol/L in EBM; control: DMSO), n = 3. ANOVA, Fisher test, Mean with 95% CI, #$P < .05$ with vs without blood. *$P < .05$ with vs without 1,25-VitD3.