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The effects of vitamin D deficiency on cerebral circulation

PhD thesis

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List of Abbreviations

1,25(OH)$_2$D: 1,25-dihydroxyvitamin D

25(OH)D: 25-hydroxyvitamin D

AACA: azygous anterior cerebral artery

ACA: anterior cerebral artery

AR: androgen receptor

CAO: carotid artery occlusion

CoBF: cerebrocortical blood flow

COX-2: cyclooxygenase 2

eNOS: endothelial nitric oxide synthase

IL: interleukin

MCA: middle cerebral artery

MMP: matrix metalloproteinase

NF-κB: nuclear factor-κB

NO: nitric oxide

PCOS: polycystic ovary syndrome

RAS: renin–angiotensin system

ROS: reactive oxygen species

RXR: retinoid X receptor

UTP: uridine-5'-triphosphate

TP: thromboxane prostanoid

VEGF: vascular endothelial growth factor

VitD: vitamin D

VDD: vitamin D deficiency

VDR: vitamin D receptor

VDRE: vitamin D response element

VSMC: vascular smooth muscle cell

1. Introduction

Vitamin D (VitD) is best known for its role in calcium and bone homeostasis; however, in the recent decades, studies have revealed its extraskeletal effects, including the modulation of the immune and cardiovascular systems (1-3). Accordingly, efforts have been made to examine the causes, consequences and prevention strategies of the "world pandemic" of vitamin D deficiency (VDD) (4). Although VDD has already been linked to increased risk for several diseases (4), the association between low levels of VitD and cerebrovascular disorders is still controversial, and the mechanism leading to increased incidence and severity of ischemic stroke in VDD is obscure (5).

1.1. Vitamin D biosynthesis and metabolism

VitD is a lipid-soluble vitamin that functions as a steroid hormone (6). Vitamin D can represent either vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol), both of which are produced naturally by ultraviolet B radiation (290 to 315 nm wavelength) from ergosterol in yeast and mushrooms or from 7-dehydrocholesterol in the epidermis (4). Humans acquire VitD mainly from its precursors upon exposure to sunlight, and to a lesser extent from certain foods such as oily fish (6). Following the exposure of skin to sunlight, 7-dehydrocholesterol is converted first to pre-vitamin D_3, which spontaneously isomerizes to vitamin D_3 in a thermosensitive process (7). Vitamin D_2 or D_3 from ingested food is incorporated into chylomicrons followed by absorption into the lymphatic system and entering the venous blood. Inactive vitamin D (as well as its metabolites) circulates within the blood stream bound to carrier proteins, mainly to the VitD binding protein (8), and subsequently it is metabolized in two steps to its active form, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, calcitriol, Figure 1B). First, the biomarker of VitD status, 25-hydroxyvitamin D (25(OH)D, Figure 1A) is produced mostly by CYP2R1 (but also by CYP27A1, CYP3A4 and CYP2J3) in the liver (4). Thereafter, $1,25(OH)_2D$ (Figure 1B) is formed exclusively by CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase), particularly in the kidney (7, 9). In addition to the kidney, many extrarenal tissues express CYP27B1, therefore those are also capable of producing the active form of VitD (10). The extrarenal production of $1,25(OH)_2D$ is stimulated mainly by cytokines and appears to be important in the paracrine regulation of cell function (11). Unlike extrarenal CYP27B1, renal 1α-hydroxylase is tightly regulated by the parathyroid hormone, fibroblast growth factor 23

as well as by plasma levels of 1,25$(OH)_2$D, calcium and phosphate ions (7, 10). In order to avoid accumulation of 1,25$(OH)_2$D or 25(OH)D, the target cells of VitD express CYP24A1 (24-hydroxylase), which converts 1,25$(OH)_2$D to biologically inactive calcitroic acid (7), whereas in the kidney, 24-hydroxylase catabolyses 25(OH)D when a sufficent amount of 1,25$(OH)_2$D has already been produced (9).

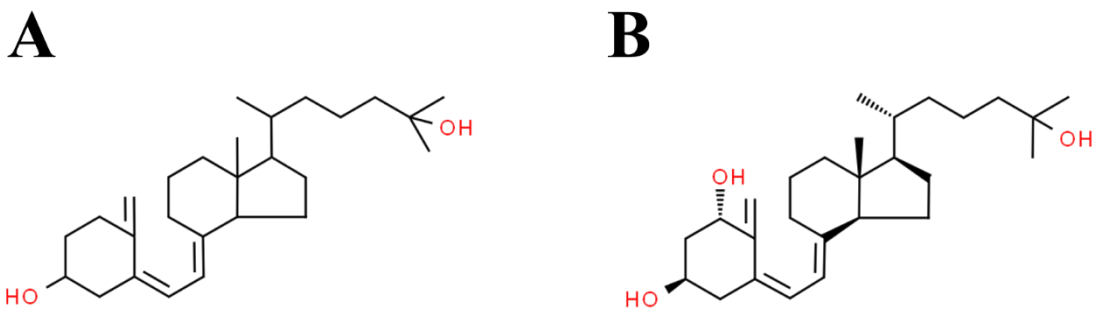

Figure 1. (A) Chemical structure of 25-hydroxyvitamin D (25-hydroxycholecalciferol, calci(fe)diol), the biomarker of vitamin D status (12) and (B) the active form, 1,25-dihydroxyvitamin D (1,25-dihydroxycholecalciferol, calcitriol) (13).

1.2. Mechanism of action of vitamin D

1.2.1. Genomic actions

The biological actions of 1,25$(OH)_2$D are mediated by the vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily and acts as a ligand-activated transcription factor (8). VDR was first discovered in chicken intestine (14), but was later found to be present in almost all cells and tissues (15). VDR regulates the expression of numerous genes the promoters of which contain specific DNA sequences known as vitamin D response elements (VDRE) (6, 15). The binding of 1,25$(OH)_2$D to VDR induces conformational changes in the receptor that facilitates its interaction with the retinoid X receptor (RXR) and subsequently the formation of a VDR/RXR heterodimer, which provides adequate DNA binding affinity (8, 16). The ligand-bound VDR/RXR heterodimeric complex binds to the VDRE on the target genes and acts as a transcription factor that up- or downregulates their transcription (8, 17). The action of 1,25$(OH)_2$D depends, however, on the involvement of tissue specific co-factors, for instance, the

steroid-specific coactivators, and subsequently on the formation of transcriptional complexes (15). Figure 2 shows schematically the genomic actions of $1,25(OH)_2D$.

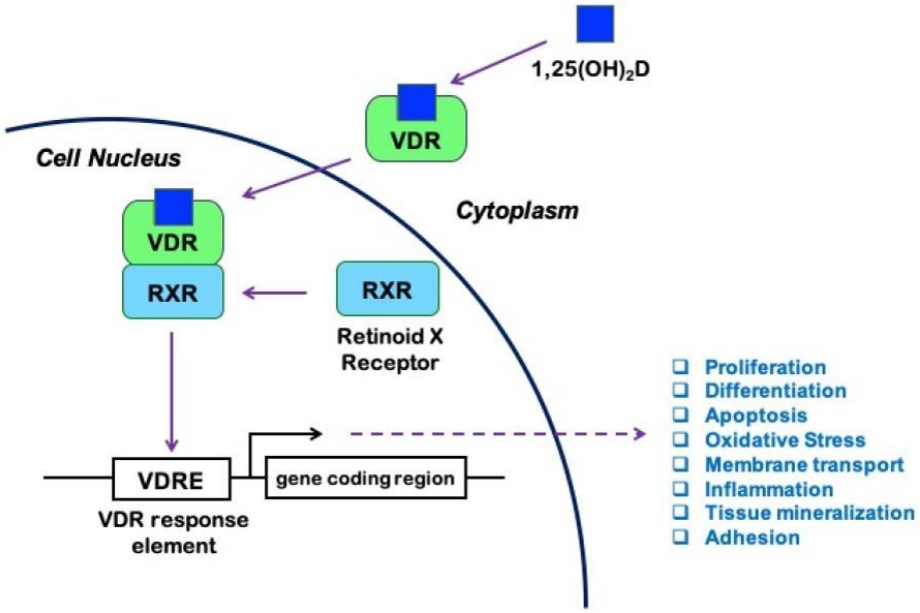

Figure 2. Genomic actions of vitamin D. 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) binds to the vitamin D receptor (VDR) and promotes its heterodimerization with the retinoid X receptor (RXR). The ligand-bound VDR/RXR complex binds to the vitamin D response elements (VDRE) in the promoters of numerous genes and modulates their transcription. Therefore, $1,25(OH)_2D$ regulates several physiological processes such as cell proliferation, differentiation and inflammation (5). Figure adopted from Kim et al. (5).

1.2.2. Non-genomic actions

Interestingly, VitD has been reported to have some rapid actions which are unlikely to involve the direct regulation of gene expression. These effects may rather be mediated by a membrane-associated VDR which has been less well characterized than the nuclear VDR (15). The non-genomic actions of VitD include the activation of signaling molecules (e.g. phospholipase C, phospholipase A_2, phosphatidylinositol-3-kinase), the rapid generation of second messengers such as Ca^{2+}, the activation of protein-kinases, as well as the opening of Ca^{2+} and Cl^- channels (18). Surprisingly, however, the rapid, non-genomic actions appear to require the presence of the nuclear VDR, implying cooperation between the membrane-associated and the nuclear VDRs (15). In addition, the ligand-bound nuclear VDR has been reported to have non-classical, non-genomic actions. In that

case, VitD regulates the target gene expression *via* protein-protein-interactions instead of binding to the VDRE (18).

1.3. Physiological significance of optimal vitamin D status

1.3.1. Determinants of vitamin D status

Vitamin D deficiency and insufficiency – defined by most experts as 25(OH)D levels below 20 ng/mL (50 nmol/L) and within the range of 21–29 ng/mL (52.5–72.5 nmol/L, respectively (2)) – affect approximately 1 billion people worldwide (4). Lifestyle and environmental factors which limit sunlight exposure of the skin are the main causes of VDD, but the decreased synthesis of 25(OH)D or $1,25(OH)_2D$, and heritable disorders such as hereditary VitD-resistant rickets could also reduce the bioavailability of VitD (4). Since only few foods contain naturally VitD (like cod liver oil, shiitake mushrooms, egg yolk), in general, sufficient VitD supply can be provided only by exposure to sunlight or by taking VitD supplements (3, 4). As a variety of factors could reduce the cutaneous production of VitD such as ultraviolet protection, increased skin pigmentation, age, seasonal and geographical variation (3), it is recommended to take VitD supplements: in general, 1000–2000 IU/day are needed to reach and maintain 25(OH)D levels greater than 30 ng/mL in most of the healthy population in order to prevent VDD (2). Unlike VDD, VitD intoxication (25(OH)D levels higher than 150 ng/mL (374 nmol/L)) is extremely rare, particularly because it cannot be caused by exposure to sunlight, since excess pre-vitamin D_3 or vitamin D_3 is destroyed by sunlight itself (4).

1.3.2. Physiological role of vitamin D

VitD appears to control the expression of more than 200 genes as well as several signaling molecules and second messengers, including those not typically associated with mineral homeostasis (16, 18). The active form of VitD regulates, for instance, cellular proliferation, differentiation, apoptosis, angiogenesis, oxidative stress, membrane transport, matrix homeostasis, cell adhesion, immune functions, insulin secretion, and renin expression (4, 16, 18, 19), thus VitD has an integral physiological role in nonskeletal tissues. Consequently, in addition to its well-characterized roles in calcium and phosphate homeostasis as well as in bone metabolism, VitD exerts beneficial effects, for instance,

on glucose homeostasis, the immune response and the cardiovascular system (4). Accordingly, VDD is associated with increased risk for cancer (colon, prostate and breast), diabetes mellitus, metabolic syndrome, infections, autoimmune diseases, depression, schizophrenia, and cardiovascular diseases (4, 20). Nevertheless, VDD impairs the mineral and bone homeostasis characterized by rickets and growth retardation in children, as well as osteomalacia, osteoporosis and decreased muscle strength or sarcopenia in adults and elderly (4).

Although VitD intoxication is rare, it can be caused by taking extensively high doses of VitD supplements (4). The clinical manifestations of VitD toxicity are related primarily to hypercalcemia, and they include confusion, depression, psychosis, gastrointestinal disorders, renal failure and cardiovascular symptoms such as hypertension and bradyarrhythmias (21). Surprisingly, it appears that both low and high 25(OH)D levels are associated with increased risk of total (22) and cardiovascular mortality (23, 24), implying a U-shaped association between VitD concentrations and health. Although VitD appears to have a broad therapeutic window, the latter still has to be defined, especially for preventing cardiovascular and cerebrovascular diseases (19). Nevertheless, optimal VitD supply is considered to be a prerequisite for health in all age groups (25).

1.4. Impacts of vitamin D on the vascular system

1.4.1. Effects of vitamin D on the cardiovascular system

There is a growing body of evidence linking VDD to cardiovascular diseases (6). For instance, VDD is associated with atherosclerosis, hypertension, cardiac hypertrophy, cerebrovascular diseases, coronary heart disease and peripheral artery disease (4) as well as with several cardiovascular risk factors such as dyslipidemia, insulin resistance, diabetes mellitus and abdominal obesity (4, 6, 20). VitD exerts a direct effect on the cardiovascular system, since VDRs have been found in cardiomyocytes (26), vascular smooth muscle cells (VSMCs) (27), endothelial cells (28), circulating monocytes, macrophages, dendritic cells, activated T cells (29), and platelets (30). Furthermore, CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase) is expressed in most of those cells, which enables local synthesis of $1,25(OH)_2D$ (6). The cardiovascular protective effects of VitD include the modulation of immune, inflammatory and endothelial functions (6).

Furthermore, VitD regulates cell proliferation and migration, renin expression, extracellular matrix homeostasis, and it may attenuate the adverse effects of advanced glycation end products on endothelial cells (6, 20). In addition, VitD has antithrombotic effect, since it downregulates tissue factor, plasminogen activator inhibitor-1 and thrombospondin-1, whereas it upregulates thrombomodulin expression in monocytes and VSMCs (6, 31). Furthermore, VitD inhibits formation of foam cells and cholesterol uptake by macrophages, thus it also exerts antiatherogenic effects (20). Figure 3 summarizes the effects of VitD related to the cardiovascular system.

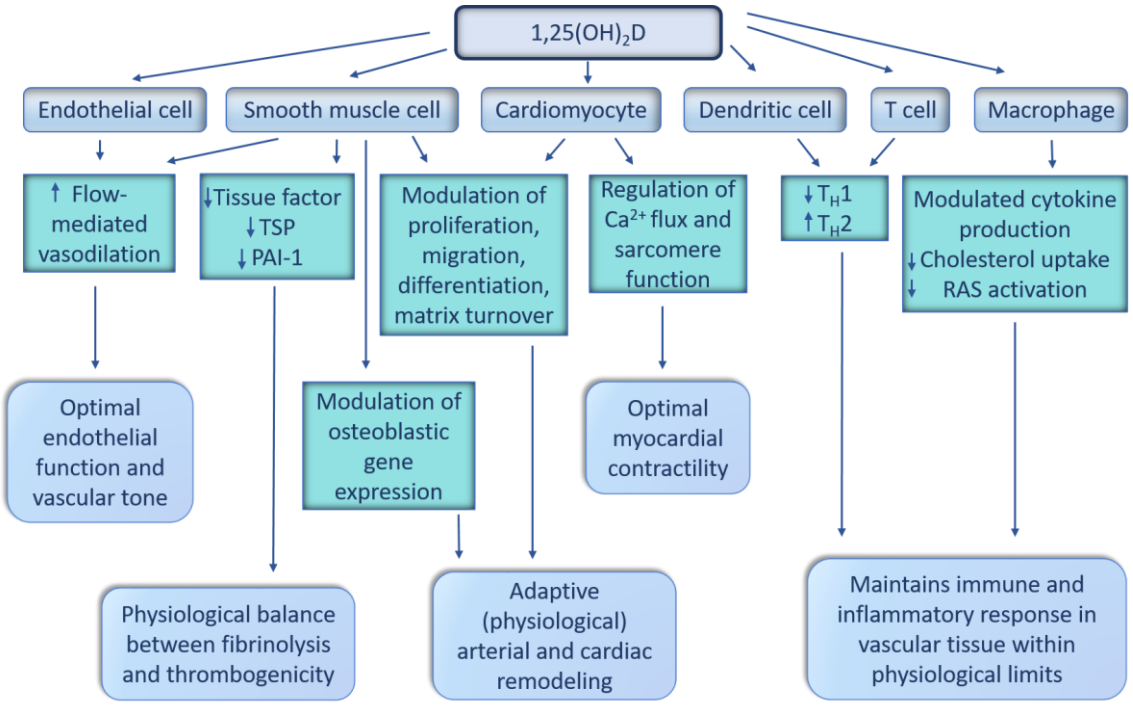

Figure 3. Overview of the cardiovascular system-related impacts of vitamin D (TSP: thrombospondin, PAI-1: plasminogen activator inhibitor-1, RAS: renin-angiotensin system, T_H1: T helper type 1 cell, T_H2: T-helper type 2 cell) (6).

1.4.2. Impacts of vitamin D on blood pressure and cardiomyocyte function

VitD appears to have a beneficial effect on arterial blood pressure, consequently VDD is linked to hypertension (6). However, the association between VitD levels and blood pressure values is not fully confirmed (32), especially in young healthy subjects (33, 34). Nevertheless, the impact of VitD on blood pressure has been attributed particularly to the negative regulation of the renin–angiotensin system (RAS) (35), since

VitD appears to decrease the activity of the cyclic adenosine monophosphate response element in the renin gene promoter (36). Consequently, VDR deficiency increases the expression of renin, therefore the production of angiotensin II which can result in hypertension and cardiac hypertrophy (35). Surprisingly, however, normotensive VDR knockout mice also developed cardiac hypertrophy (26) which could imply that VitD acts directly on cardiomyocytes (19, 37). Accordingly, VitD has been reported to stimulate cardiomyocyte relaxation, which could improve coronary perfusion during diastole, and it also regulates the extracellular matrix gene expression profile in the heart (20).

1.4.3. Effects of vitamin D on angiogenesis and vascular remodeling

VitD has been reported to regulate the expression of several genes involved in cell proliferation and differentiation (38) as well as in extracellular matrix homeostasis (6), therefore VitD appears to participate in the regulation of angiogenesis and vascular remodeling. For instance, VitD attenuates vascular remodeling (particularly prevents the changes in lumen area and lumen/wall area ratio) in intrarenal arteries in kidney fibrosis (39), and it also hinders basilar artery remodeling after subarachnoid hemorrhage in rats (40). Accordingly, VDD has been reported to decrease the lumen and increase the wall thickness of coronary arterioles of female rats (41). Altered VSMC migration and proliferation may be responsible for the vascular remodeling in VDD (24), although the literature is controversial about the effect of VitD on VSMCs. Some studies report enhanced migration and proliferation (42, 43), whereas others found VitD-induced inhibition of VSMC growth (27, 44). The effect of VitD on VSMCs appears to depend on the applied dose. For instance, at physiological doses, VitD inhibits VSMC proliferation (24) *via* blunting c-myc RNA induction (43), up-regulating the negative modulators of cell proliferation including transforming growth factor β (45) or decreasing cyclin-dependent kinase 2 activity (46). In addition, VitD participates in the modulation of endothelial cell proliferation and matrix homeostasis due to the regulation of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) (6). VEGF is known to stimulate endothelial cell proliferation and migration, and mediate vascular growth and angiogenesis (47), whereas MMPs regulate angiogenesis and vascular remodeling by degrading extracellular matrix proteins (48). In VDR deficiency, decreased expression of tissue inhibitors of MMP-1 and MMP-3, but upregulated

expression of MMP-2 and MMP-9 have been reported (6). Those alterations in extracellular matrix homeostasis may contribute to the development of vascular calcification (49). Furthermore, VitD induces the upregulation of VEGF in endothelial progenitor cells (50) as well as in mature endothelial cells (51), and also in VSMCs (17, 42) implying the role of VitD in vasculogenesis, angiogenesis and endothelial repair (47). Surprisingly, however, VitD has also been reported to inhibit angiogenesis partly *via* reducing the protein expression of VEGF in various human tumor cells (52). Thus, the effect of VitD on angiogenesis is ambiguous; however, inhibitory impact of VitD on VEGF expression and angiogenesis has been reported almost exclusively in cancer studies (53). Furthermore, VitD appears to stimulate the ability of multipotent mesenchymal stromal cells to promote vasculogenesis (54). In addition, VitD regulates the elastin and collagen content of the vessel wall (55), thus it influences vascular resistance and arterial stiffness (56). For instance, Andrukhova et al. reported increased collagen and decreased elastin content of the ascending aorta of 9-month-old VDR-deficient mice; however, they did not observe any changes in younger animals (55). Correspondingly, Salum et al. found that VitD could preserve the structure of elastic fibers and the ratio of elastic fibers to collagen in tunica media of aorta in experimental diabetes (57). Since the increase in collagen to elastin ratio could increase arterial stiffness (56), VitD appears to participate in the maintenance of normal elasticity of the vessel wall.

1.4.4. Impact of vitamin D on endothelial function

1.4.4.1. Vitamin D and the nitric oxide system

Low levels of VitD are associated with diminished flow-mediated vasodilation, which could be attributed to the endothelial dysfunction characteristic for VDD (20). Additionally, endothelial VDR appears to play an important role in preserving endothelial function (58). VitD has been reported to upregulate the expression of endothelial nitric oxide synthase (eNOS) (59), to improve the dimer to monomer ratio of the eNOS protein (60), and it also modulates the phosphorylation of eNOS (40, 61) leading to increased eNOS activity, and thus to enhanced nitric oxide (NO) production (62). Furthermore, VitD appears to mediate intracellular kinases such as the cyclic adenosine monophosphate-activated protein kinase (40), the phosphoinositide-3-kinase/Akt, p38

mitogen-activated protein kinase (MAPK), and the extracellular signal-regulated kinase (ERK)/MAPK pathways, regulators of endothelial NO production (61). In animal experiments, VitD treatment increased eNOS protein expression, leading to improved endothelial-dependent relaxation (63). In VDR-deficient mice, the impaired endothelium-mediated vasodilation of the aorta was accompanied by reduced eNOS expression (58). Additionally, diminished NO production has been reported in mice with functionally inactive VDR (55), and endothelial dysfunction in resistance arteries of rats exposed to early-life VDD (64).

1.4.4.2. Oxidative stress, inflammation and vitamin D

Endothelial function can be compromised by oxidative stress, inflammation and atherogenic processes (24, 65), all of which might be associated with VDD (6). Reactive oxygen species (ROS) induce pro-inflammatory cytokine secretion and, *vica versa*, pro-inflammatory cytokines increase ROS production (65). VDD is associated with the increased production of ROS and pro-inflammatory mediators in the cardiovascular system, which could contribute to the development of endothelial dysfunction (6). The protective role of VitD is exerted through the upregulation of the antioxidant, radical scavenging enzyme, the cytosolic copper-zinc superoxide dismutase (CuZn-SOD) (51, 66) as well as by decreasing the expression of the free radical generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme and its subunits in endothelial cells (60, 66). Consequently, VitD-deficient diet has been reported to cause an increase in superoxide anion production in the aortic wall (67). Increased ROS level could lead to inactivation of NO or oxidation of tetrahydrobiopterin, a critical cofactor for eNOS, which leads to eNOS uncoupling, and thus, to endothelial dysfunction (68).

VitD appears to suppress inflammation *via* several mechanisms, such as inhibition of prostaglandin and cyclooxygenase pathways, upregulation of anti-inflammatory cytokines (interleukin (IL)-4 and IL-10), downregulation of pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-23, tumor necrosis factor-α (TNF-α), and interferon-γ), decreasing cytokine-induced expression of adhesion molecules, and downregulation of the RAS (20) (6). The lack of VitD-mediated renin suppression in VDD leads to an increase in angiotensin II levels (35), which can promote vascular inflammation (65). VitD has been reported to suppress the expression of inflammatory mediators such as TNF-α,

cyclooxygenase 2 (COX-2) and monocyte chemoattractant protein-1 in the aorta of ApoE-deficient atherosclerotic mice (69). Additionally, VitD downregulated the expression of COX-2 and the thromboxane prostanoid (TP) receptor in renal artery segments and aortic endothelial cells of ovariectomized rats, and thus improved endothelial function (70). Furthermore, VitD inhibits the activation of nuclear factor-κB (NF-κB) (71), and it decreases the expression of IL-6 in endothelial cells (72), thus it prevents endothelial inflammation, improves flow-mediated vasodilation, and protects against atherosclerosis (6). In addition, VitD suppresses the responses of T helper type 1 (T_H1) and T helper type 17 (T_H17) cells, whereas it supports regulatory T (T_{reg}) and T helper type 2 (T_H2) cells, which also contributes to the prevention of atherosclerosis (6, 73, 74).

1.4.5. Effects of vitamin D on the vascular tone

As VitD influences endothelial function, especially NO bioavailability (6), it is not surprising that VitD contributes to the regulation of vascular tone. For instance, enhanced myogenic tone (64) and increased angiotensin II-induced vasoconstriction (75) of mesenteric arteries have been reported in VDD. On the contrary, in coronary arteries of female rats, VDD reduced the myogenic as well as the thromboxane A_2 agonist evoked tone (41). In addition to modulation of NO production, VitD appears to reduce the endothelium-dependent contraction of the aorta of spontaneously hypertensive rats due to reducing calcium influx into the endothelial cells, thereby decreasing the production of endothelium-derived contracting factors (76, 77). Furthermore, VitD normalized the vascular reactivity of mesenteric arteries of spontaneously hypertensive rats *via* restoring the function of apamin- and ATP-sensitive K^+ channels in VSMCs (78).

1.5. Vitamin D deficiency and cerebrovascular diseases

1.5.1. Cerebrovascular disorders

In order to provide continuous oxygen and nutrient supply for neurons, the cerebral circulation is tightly controlled by myogenic, metabolic, endothelial, and neuronal mechanisms (79). Notably, the cerebral blood flow remains constant despite fluctuations in arterial blood pressure within the range of 60–150 mmHg due to autoregulation (79). However, when blood pressure is not within the limits of autoregulation, there is a risk of

brain injury (80). Additionally, the cerebrovascular tone is influenced by arterial pCO_2 and, to a lesser extent, also by pO_2. Importantly, hypoxia and hypercapnia promote the release of vasoactive mediators from cerebral vessels, and the subsequent vasodilation increases the blood flow, and thus tissue oxygenation (79). Since the central nervous system is highly vulnerable, the impairment of cerebral autoregulation is likely to lead to neurological disorders (80, 81). It is well-known that cerebrovascular diseases, particularly ischemic stroke, belong to the leading causes of death and disability worldwide (81, 82). Strokes due to atherosclerosis of a larger artery account for approximately one third of all stroke cases (83). For instance, carotid artery atherosclerosis may lead to ischemia as a result of distal embolization, or due to the hypoperfusion of brain tissue supplied by the severely stenotic or occluded vessel (83). Unilateral carotid artery occlusion (CAO) in mice has been reported to induce rapid but transient reductions in the cerebrocortical blood flow (CoBF) of the ipsilateral hemisphere, since the CoBF returns close to the baseline level within 30 sec (84). The rapid cerebrovascular adaptation could be attributed to efficient compensatory pathways – supplied primarily by the contralateral carotid artery – including large vessels of the Willis circle and smaller pial anastomoses between the terminal branches of the anterior, middle and posterior cerebral arteries (84-86). The number and diameter of those anastomoses appear to influence the perfusion of the penumbral cortex, in particular following occlusion of an artery distal to the Willis circle (87, 88). Accordingly, the extent of the native pial collateral circulation in mice has been reported to predict the severity of ischemic stroke (89).

1.5.2. Cerebrovascular impacts of vitamin D deficiency

VDD is particularly frequent in people who have suffered stroke, which is attributed to their limited mobility, advanced age or malnutrition (i.e. conditions leading to decreased bioavailability of VitD) (5). Additionally, observational studies imply that VDD is associated with the increased risk of cerebrovascular diseases including ischemic stroke (5, 90-94), chronic brain injury associated with cerebral small vessel disease (95), cerebral cavernous malformation disease (5), vascular dementia (96, 97), and increased arterial stiffness related cognitive impairment (98). Furthermore, VDD has been linked to poor post-stroke outcome (99), for instance, more severe cognitive impairment (5), and it

is also associated with higher risk of death at one or two years following stroke, and with greater risk of early recurrent stroke (5). Although VDD appears to increase the risk for cerebrovascular diseases (5), large Mendelian randomization studies have failed to provide evidence for causal association between 25(OH)D levels and ischemic stroke (100-102). Thus, the beneficial effect of VitD supplementation on reducing the incidence and severity of stroke is still questionable (5, 90). Similarly, animal studies investigating the role of VitD in the outcome of stroke are also controversial. For instance, VDD has been reported to increase the infarction volume, exacerbate the behavioral impairment, and compromise the blood-brain barrier after cerebrovascular occlusion (103, 104). Furthermore, VitD supplementation reduced the ischemia-induced brain damage in rodent brains (105, 106). On the contrary, VDD did not affect the extent of brain injury following ischemic stroke (107). Thus, the impact of VDD on the cerebrovascular system remains unclear.

Nevertheless, VDD appears to increase the risk and worsen the severity of ischemic stroke *via* direct and indirect mechanisms. The direct effects of VDD include enhanced platelet aggregation, upregulation of tissue factor expression, downregulation of antithrombin and thrombomodulin expression, impaired biosynthesis of neurotrophic factors and neurotransmitters, and compromised detoxication pathways of the brain (90). Since VDD is associated with several risk factors for stroke, it may also increase the incidence of cerebrovascular diseases indirectly (90). For instance, VDD is linked to hypertension (6), which is ultimately one of the major modifiable risk factors for cerebral ischemia (81). Diabetes mellitus and insulin resistance are also associated with VDD, which may be attributed to the impaired β-cell function and insulin sensitivity of the target cells in VDD (108). VDD stimulates the secretion of parathyroid hormone, and thus it results in secondary hyperparathyroidism (109). Since elevated parathyroid hormone levels have been found in stroke patients, an association between parathyroid hormone levels and cerebrovascular diseases is presumable (109). Furthermore, VDD favors inflammation (6), which may play a central role in the pathogenesis and progression of stroke (81). In addition, VDD has been associated with subclinical carotid atherosclerosis (110). Since atherosclerosis, particularly that of the carotid arteries, may lead to cerebral ischemia (109), VitD is likely to prevent stroke events partly by being protective against atherosclerosis (24). Although VitD is known to influence several physiological

processes relevant to vascular homeostasis, its effect on cerebrovascular morphology and function is still unrevealed.

1.5.3. Androgens and vitamin D in the cerebral circulation

Androgen excess is likely to increase the incidence of cerebrovascular diseases, since hyperandrogenic women are at increased risk for stroke as compared to premenopausal healthy women (111, 112). Hyperandrogenic disorders, for instance, polycystic ovary syndrome (PCOS), are associated with endothelial dysfunction and vascular remodeling (113, 114). Importantly, in addition to hyperandrogenemia, insulin resistance and chronic subclinical inflammation – characteristics of PCOS – contribute to the increased vulnerability to vascular disorders (113, 114). Additionally, men are reported to have a higher incidence of cerebrovascular diseases, whereas the severity of outcome is greater in age-matched women (5, 112). In addition to genetic predisposition and lifestyle, the actions of sex steroids may contribute to the aforementioned sex differences (112). Sex steroids have considerable impact on cerebral circulation: Endogenous and exogenously administered gonadal hormones influence the cerebrovascular tone and blood perfusion under physiological and pathophysiological conditions (112, 115). While the impact of estradiol on the cerebral circulation is well known, the effect of testosterone on cerebral vessels is more obscure (112, 115), although the presence of androgen receptors (ARs) in the endothelium and VSMCs of cerebral vessels has already been demonstrated (111, 115, 116). In general, testosterone modulates vascular reactivity by genomic and non-genomic actions involving both AR-dependent and -independent mechanisms (112). In rodent models, long-term treatment with androgens results in increased vascular tone in female (117) as well as in male orchiectomized rats (118) possibly due to decreasing the production of endothelial-derived hyperpolarizing factor and increasing thromboxane A_2 synthesis (115). However, other studies reported that androgens at physiological doses inhibit oxidative-stress-induced platelet aggregation (119) and suppress the upregulation of COX-2 and hypoxia-inducible factor-1 in male rats (120), indicating an antithrombotic and anti-inflammatory effect. Correspondingly, low testosterone levels in men appear to increase the risk for stroke (121) and carotid atherosclerosis (122), a common cause of ischemic injury of brain. These findings imply the protective role of endogenous androgens in men.

According to the literature, VDD might influence the vascular effects of androgens and *vica versa*. For instance, endogenous sex steroids appear to affect the impact of VDD on endothelial vasodilation in mesenteric arteries (64), and an interplay between androgens and VitD has been reported in the regulation of vascular cell proliferation (123), which could be attributed to a cross-talk between AR- and VDR-mediated gene expression (124, 125). Interestingly, 67–85% of women with PCOS are affected by VDD (126). Moreover, VitD influences the development of PCOS due to the modulation of gene transcription and hormonal regulation (126, 127). In addition, VDD may worsen the cardiovascular manifestation of PCOS (126). Accordingly, VitD supplementation has been reported to restore the contractility of aorta (117) and the tone of gracilis muscle arterioles (128) in hyperandrogenic female rats. On the contrary, VitD treatment failed to improve the diminished endothelial-dependent relaxation induced by androgen excess in resistance arteries (129). Although both VDD and hyperandrogenism appear to be associated with cerebrovascular disorders (5, 130), their combined effect on cerebral arteries has not been examined before.

2. Objectives

Although vitamin D deficiency has been associated with increased risk and severity of cerebrovascular diseases including ischemic stroke, the impact of vitamin D on the cerebrovascular system and thus, on cerebrovascular adaptation to ischemia has not been examined before. Furthermore, the increased incidence of cerebrovascular disorders in men and in hyperandrogenic women as compared to premenopausal healthy women has been proven by several studies; however, the role of testosterone in the cerebrovascular manifestation of vitamin D deficiency has not been revealed previously.

In our experiments, we aimed at investigating:

- the impact of vitamin D deficiency on the morphological, biomechanical and functional properties of cerebral arteries,
- the interplay between testosterone and vitamin D deficiency in the remodeling of cerebral arteries,
- the role of vitamin D signaling in cerebrovascular adaptation to unilateral carotid artery occlusion laying emphasis on the pial collateral circulation.

3. Results

3.1. Vitamin D deficiency-induced morphological and functional alterations of cerebral arteries in male rats

In order to investigate the cerebrovascular effects of VDD, anterior cerebral artery (ACA) segments of male rats either receiving VitD-deficient diet (VDD group) or supplied with VitD (Control group) were isolated and excised. The morphological, biomechanical and functional properties of arteries were examined using pressure microangiometry. Hematoxylin and eosin, resorcin-fuchsin, and smooth muscle actin staining were used to determine wall thickness, elastic fiber density, and smooth muscle cell counts in the vessel wall, respectively. In addition, sections were immunostained for eNOS, COX-2 and androgen receptor (AR). Physiological parameters and hormone levels of the rats were also measured.

3.1.1. Effects of vitamin D deficiency on the physiological parameters of rats

At the end of the 8-week-long VitD deprivation, the physiological parameters of the rats were determined. In order to assess the efficacy of VitD deprivation, serum 25(OH)D levels were measured, and they found to be significantly lower in VDD as compared to control animals at the 8^{th} week of treatment (Table 1). The body weight, heart/body weight ratio, testis weight, mean arterial blood pressure, heart rate, as well as serum testosterone, androstenedione and progesterone levels did not differ between the VitD-deficient and Control groups (Table 1), indicating that the VitD-deficient diet, at least within 8 weeks, does not affect these parameters. Similarly, blood glucose levels before and after oral glucose administration did not show any difference between the two groups (Table 1). These findings exclude the possibility that any morphological or functional changes of the cerebral arteries would be secondary consequences of VDD-induced hypertension, metabolic syndrome or altered androgenic hormone status.

Table 1. Physiological parameters and serum levels of hormones, 25(OH)D and glucose. VDD did not influence either the physiological parameters and serum hormone levels of rats or the serum levels of glucose during the oral glucose tolerance test (OGTT). The VitD-deficient diet induced significantly lower serum 25-hydroxyvitamin D (25(OH)D) level (****p<0.0001, Student's unpaired t-test). All parameters except for blood glucose levels were measured at the 8th week of treatment. OGTT was performed at week 6. Data are presented as mean ± SEM.

MEASURED PARAMETER	**CONTROL** n=11	**VDD** n=11
Body weight *(g)*	435.7±17.7	444.5±10.3
Heart/body weight *(%)*	0.34±0.01	0.34±0.01
Testis weight *(g)*	3.74±0.16	3.71±0.33
Mean arterial blood pressure *(mmHg)*	131±4	134±4
Heart rate (*1/min*)	357±18	348±11
Serum testosterone *(ng/mL)*	6.56±0.84	5.94±0.91
Serum androstenedione *(ng/mL)*	0.57±0.14	0.54±0.12
Serum progesterone *(ng/mL)*	14.42±2.31	19.86±2.61
Serum 25(OH)D *(ng/mL)*	19.66±0.81	3.59±0.21 ****
Glucose (OGTT 0 min) (*mmol/L)*	6.24±0.46	5.49±0.20
Glucose (OGTT 60 min) (*mmol/L)*	7.61±0.33	7.59±0.37
Glucose (OGTT 120 min) (*mmol/L)*	5.51±0.39	5.41±0.31

3.1.2. Cerebral arterial morphology

Excised ACA segments were examined in Ca^{2+}-free Krebs solution with pressure myograph in order to determine arterial morphology under passive conditions, as we hypothesized that VDD could cause remodeling of cerebral arteries. The wall thickness (Figure 4A) and the wall thickness/lumen diameter ratio (Figure 4B) showed a significant increase in the VDD group under passive conditions.

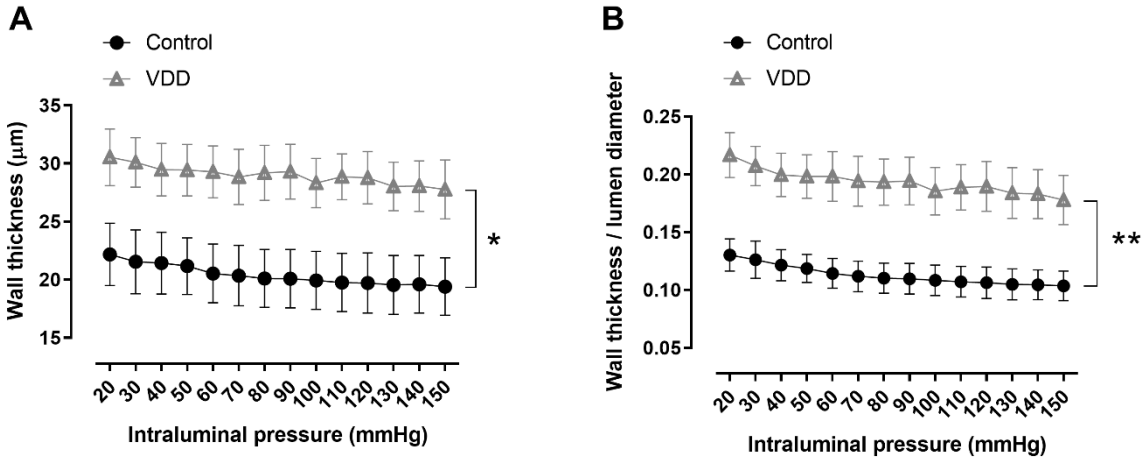

Figure 4. Remodeling of the arterial wall in VDD. (A) VDD significantly increased the wall thickness as well as (B) the wall thickness/lumen diameter ratio determined with pressure myograph (*p<0.05, **p<0.01, two-way repeated measures ANOVA followed by Bonferroni post hoc test, n=10-11). Data are presented as mean ± SEM.

Wall thickness was also determined on hematoxylin- and eosin-stained sections (Figure 5A) to confirm our findings based on pressure myography. Accordingly, VDD caused an increase in wall thickness (Figure 5B), which unambiguously indicates vascular remodeling.

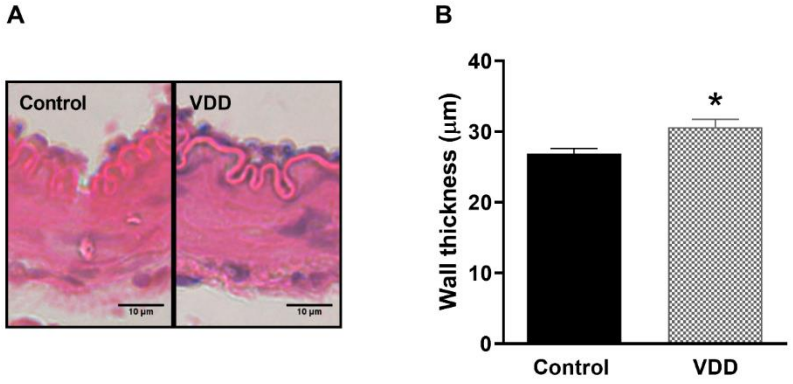

Figure 5. Increase of the arterial wall thickness in VDD. (A) Representative images of ACA sections stained with hematoxylin and eosin. (B) VDD significantly increased the wall thickness of arteries (*p<0.05, Student's unpaired t-test, n=5-7). Data are presented as mean ± SEM.

In order to identify the type of remodeling (i.e. inward, hypertrophic, etc.), the cross-sectional area of the vessel wall and lumen was determined. VDD significantly increased the wall cross-sectional area (Figure 6A) with a tendency to decrease the lumen cross-sectional area (Figure 6B), indicating the development of hypertrophic remodeling in the VDD group.

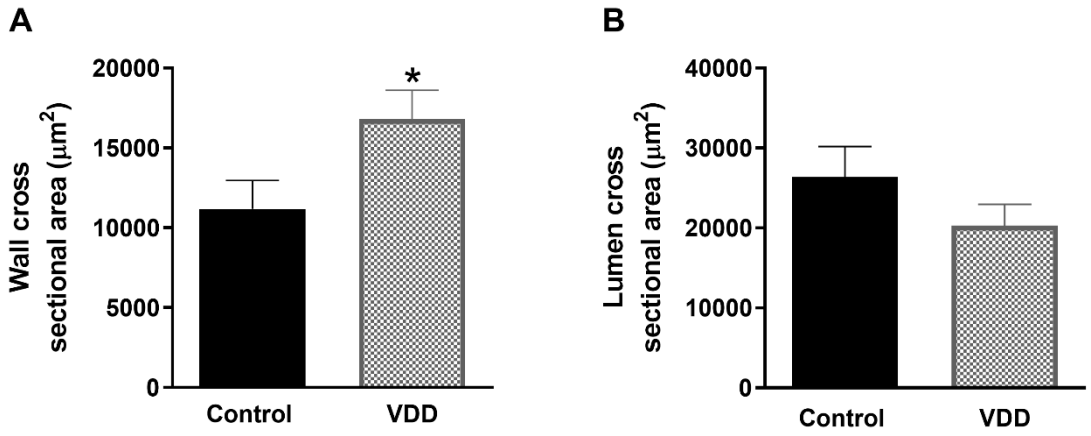

Figure 6. Cross-sectional area of the arterial wall and lumen in VDD. (A) The cross-sectional area of the vessel wall increased significantly in VDD as compared to the Control group (*$p<0.05$, Student's unpaired t-test, n=9-9). (B) VDD resulted in slightly decreased lumen cross sectional area, but the difference did not reach the level of statistical significance. Data are presented as mean ± SEM. The cross-sectional areas were determined at 50 mmHg intraluminal pressure under passive conditions.

Thereafter, in order to determine the alterations in the vessel wall responsible for hypertrophy, the thickness of the tunica media and tunica intima was evaluated on histological sections. VDD resulted in increased thickness of the tunica media (Figure 7A); however, it did not affect the thickness of the tunica intima (Figure 7B). Thus, VDD caused a decrease in the intima/media ratio of ACA (Figure 7C). Additionally, after immunohistochemical staining of smooth muscle actin, the abundance of nuclei was determined in the tunica media. The significantly increased nucleus count found in the smooth muscle layer of arteries from the VDD group (Figure 7D) indicated the presence of more vascular smooth muscle cells in the vessel wall, which could be responsible for the increased tunica media thickness and thereby for hypertrophic remodeling in the VDD group.

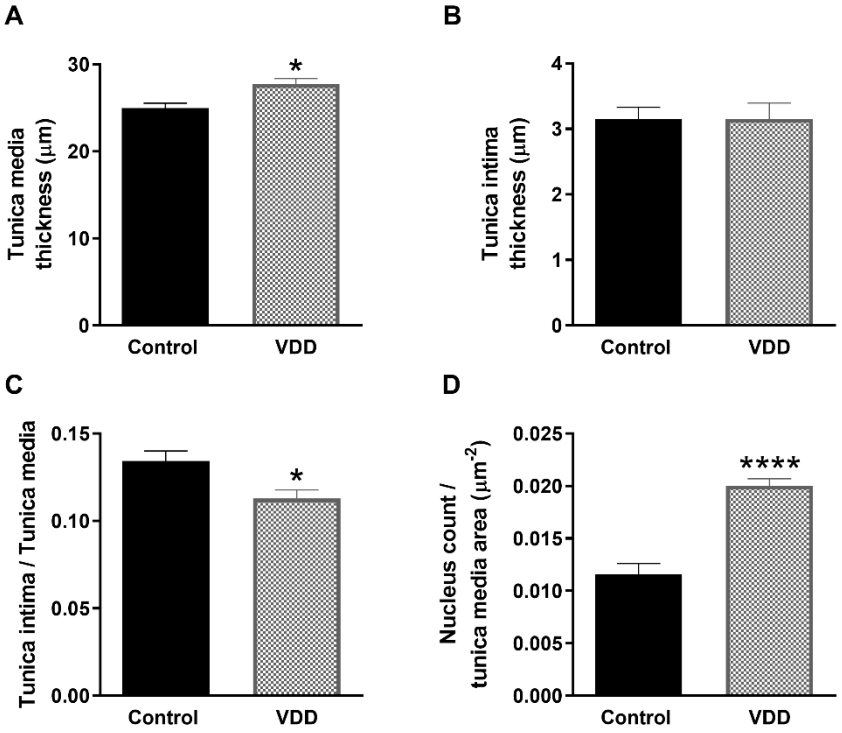

Figure 7. Morphological alterations of the tunica media in VDD. (A) VDD increased the thickness of the tunica media but (B) it did not affect the thickness of the tunica intima of cerebral arteries (*p<0.05, Student's unpaired t-test, n=4-4). (C) Accordingly, VDD decreased the intima/media ratio of arteries (*p<0.05, Student's unpaired t-test, n=4-4). (D) Significantly more nuclei were detected in the smooth muscle layer of ACAs of VDD animals as compared to controls (****p<0.0001, Student's unpaired t-test, n=4-6). Data are presented as mean ± SEM.

3.1.3. Biomechanical properties

As previously mentioned, the wall thickness/lumen diameter ratio increased in the arteries of the VDD group. In accordance with this finding, the tangential wall stress was significantly lower in the VDD group under passive conditions (Figure 8**),** as this biomechanical parameter is inversely proportional to the wall-to-lumen ratio. The incremental elastic modulus and the distensibility did not change significantly between the groups at 50 mmHg intraluminal pressure measured under passive conditions (elastic modulus: 2.89±0.27 log(kPa) and 2.65±0.19 log(kPa), distensibility: 1.41±0.12 log(Pa^{-1}) and 1.18 ± 0.23 log(Pa^{-1}) for the Control and VDD groups, respectively).

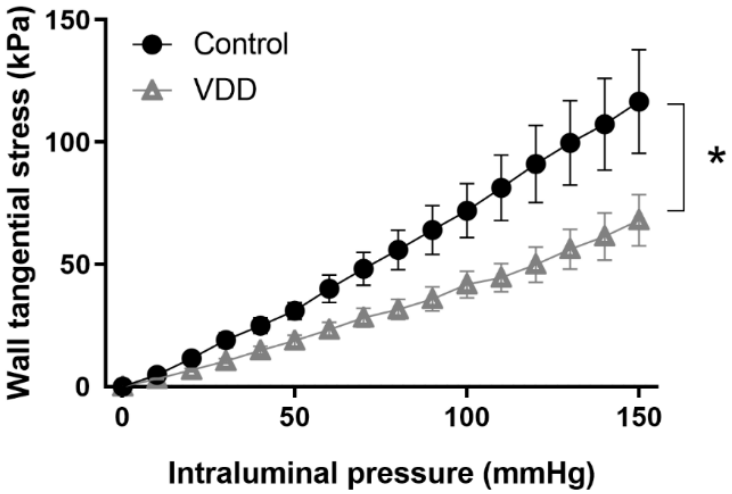

Figure 8. Decreased tangential wall stress of the cerebral arteries in VDD. VDD caused a decrease in the tangential stress of the vessel wall throughout the entire pressure range under passive conditions (*p<0.05, two-way repeated measures ANOVA followed by Bonferroni post hoc test, n=10-11). Tangential wall stress was computed according to the Laplace-equation: $\sigma_{tang}=(P*Ri)/h$, where σ_{tang} is the tangential wall stress, P is the intraluminal pressure, Ri is the inner radius, and h is the wall thickness. Data are presented as mean ± SEM.

To investigate whether VDD impacts the elastic components of the vessel wall, the density of the elastic fibers was determined after resorcin-fuchsin staining. Their density, however, did not show any difference between the groups based on the green color (suppressed by the violet of the resorcin-fuchsin dye) intensity measurements made in radial direction, outward from the luminal surface (Figure 9).

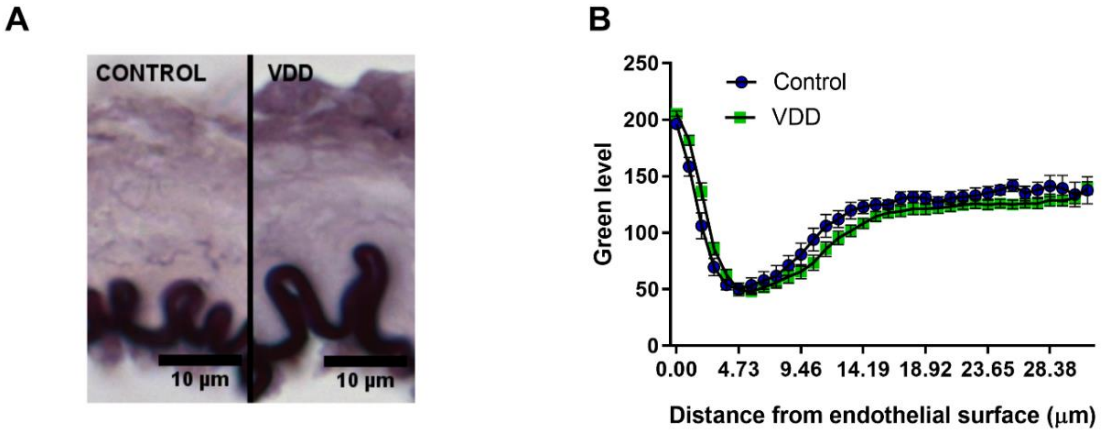

Figure 9. Unchanged elastic components of the vessel wall in VDD. (A) Representative images of cerebral arteries stained with Weigert's resorcin-fuchsin. (B) Elastic fiber density did not differ between the groups according to the measurement of green color intensity as a function of distance from the luminal surface measured in resorcin-fuchsin-stained segments (two-way repeated measures ANOVA followed by Bonferroni post hoc test, n=10-11). Lower green color intensity indicates higher elastic fiber density since the violet of the resorcin-fuchsin stain suppresses green. Data are presented as mean ± SEM.

3.1.4. Immunohistochemistry

VitD has been reported to impact the protein expression of several genes, many of which have crucial roles in the modulation of vascular reactivity (6). Therefore, eNOS, COX-2 and AR immunostaining was used to evaluate the possible role of VitD in the modulation of their cerebrovascular expression. The optical density of endothelial eNOS staining was lower in the VDD group, indicating lower expression of eNOS (Figure 10A,B). In contrast, COX-2 expression was enhanced in the endothelial layer of arteries from the VDD group (Figure 10C,D). Finally, to examine AR protein expression, the percentage of positively stained area in the vessel wall was determined. The expression of AR protein was decreased in VDD as compared to control animals (Figure 10E,F).

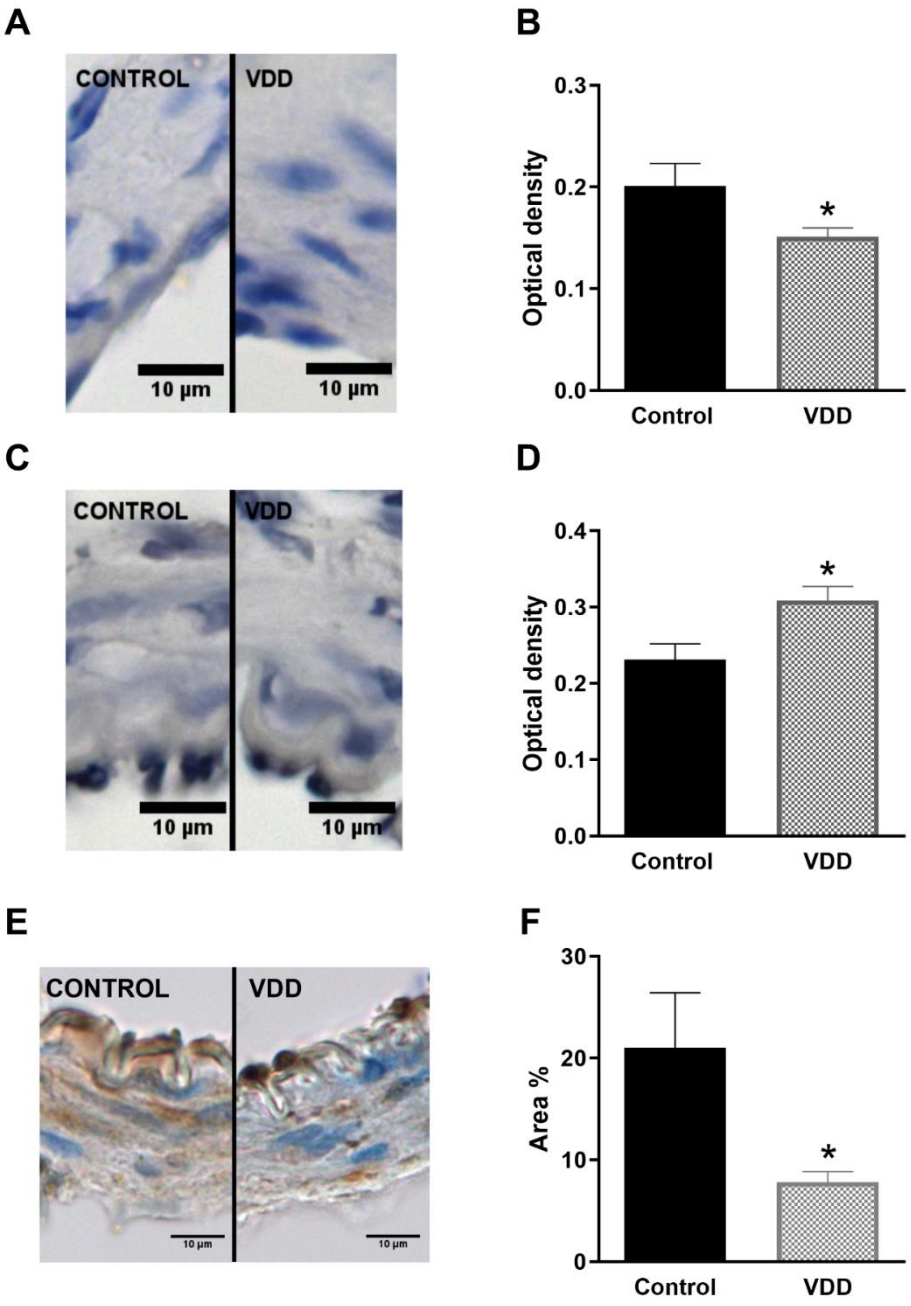

Figure 10. Altered cerebrovascular eNOS, COX-2 and AR expression in VDD. Representative immunohistochemical images of cerebral arteries stained for (A) eNOS, (C) COX-2 and (E) AR. (B) VDD caused a decrease in the expression of eNOS and (D) an increase in the expression of COX-2 in the endothelium of arteries (*$p<0.05$, Student's unpaired t-test, A: n=4-6, B: n=4-4). (F) The expression of AR was lower in the VDD group as compared to control animals (*$p<0.05$, Student's unpaired t-test, n=4-7). Data are presented as mean ± SEM.

3.1.5. Smooth muscle tone and endothelial reactivity

In order to evaluate whether the alterations in morphology and in the expression of proteins are accompanied by changes in vascular functions, the endothelium-dependent relaxation capacity (using bradykinin) as well as the myogenic and uridine-5'-triphosphate (UTP)-induced tones of ACA were determined. Bradykinin (10^{-6} mol/L) induced slight vasodilatation after precontraction in the Control group but it failed to relax the arteries from the VDD group (Figure 11A), indicating endothelial dysfunction in VitD-deficient animals. Segments from VDD animals showed a two-fold increase in myogenic tone as compared to control ones, implying that the 8-week VitD deprivation doubled the spontaneous tone of vessels (Figure 11B). Because UTP is a potent vasoconstrictor of cerebral arteries (131), the agonist-induced responsiveness with this agent was also tested. UTP (10^{-4} mol/L) caused potent constriction in both groups, but the induced tone was significantly greater in the VDD group (Figure 11C).

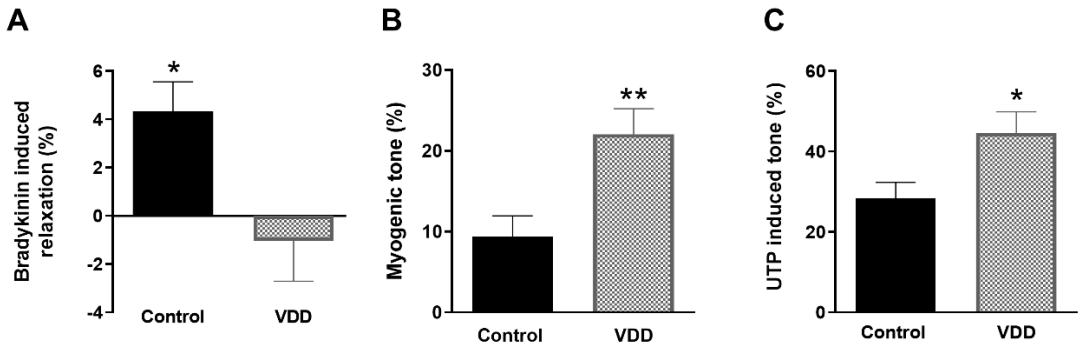

Figure 11. **Alterations of vascular reactivity induced by VDD.** (A) Bradykinin induced endothelium-dependent relaxation after precontraction in the Control group but did not relax the arteries in the VDD group (*p<0.05, Student's unpaired t-test, n=9-9). (B) ACAs possessed myogenic tone and this tone was greater in the VDD group, (C) in addition, UTP-induced contraction also increased in the VDD group (*p<0.05, **p<0.01, Student's unpaired t-test, B: n=8-8, C: n=10-9). Bradykinin-induced relaxation, myogenic tone and UTP induced tone were computed as: $T_{BK}\%=100*(Ri_{BK}-Ri_{UTP})/Ri_{UTP}$, $M\%=100*(Ri_{Cf}-Ri_{nKR})/Ri_{Cf}$, $T_{UTP}\%=100*(Ri_{Cf}-Ri_{UTP})/Ri_{Cf}$, *respectively.* Ri_{BK} and Ri_{UTP} is the inner radius after incubation with bradykinin and UTP, respectively. Ri_{Cf} is the inner radius in Ca^{2+}-free solution, whereas Ri_{nKR} is the inner radius in normal Krebs-Ringer solution. All parameters were measured at 50 mmHg intraluminal pressure. Data are presented as mean ± SEM.

3.2. Role of hyperandrogenism in the cerebrovascular manifestation of vitamin D deficiency in female rats

In order to investigate the impact of serum testosterone levels on the VDD-induced cerebrovascular alterations, we examined the ACAs of female rats receiving VitD-deficient food and/or transdermal testosterone treatment (Table 2 summarizes the experimental groups).

Table 2. Experimental groups. ♀*D*+ and ♀*D*- stand for female rats that received conventional rat chow and VitD-deficient diet, respectively. *T*♀*D*+ and *T*♀*D*- symbolize testosterone-treated females with vitamin D supply and with vitamin D deprivation, respectively.

SEX	♀			
VITAMIN D	+		–	
TESTOSTERONE TREATMENT	+	–	+	–
SYMBOL	*T*♀*D*+ (n=12)	♀*D*+ (n=12)	*T* ♀*D*- (n=11)	♀*D*- (n=11)

3.2.1. Blood pressure and ovarian histology

In addition to determining the morphology and reactivity of ACAs, blood pressure and ovarian morphology were also examined. Arterial blood pressure was not affected by either treatment: mean arterial pressure was 116.2±6.3 mmHg in females with physiological androgen status, whereas 111.1±2.0 mmHg in hyperandrogenic female rats. VitD status had no influence on blood pressure either. The ovaries of female rats were stained with hematoxylin and eosin for histological examination. Hyperandrogenic female rats had multiple small-sized primordial follicles in the ovaries (Figure 12), indicating that hyperandrogenism impairs follicle maturation independently from VitD status.

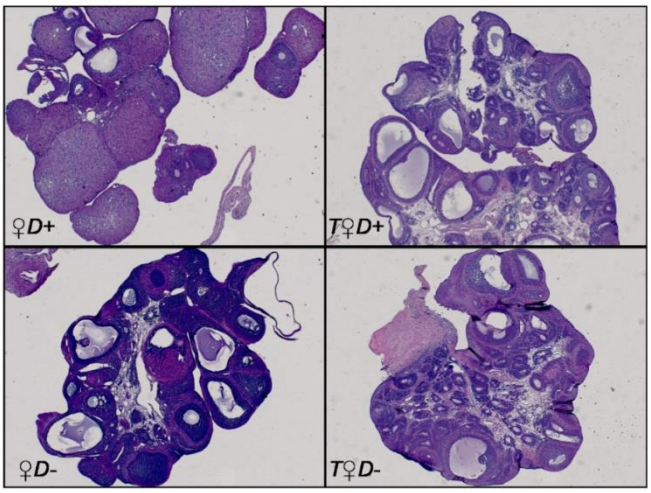

Figure 12. Representative images of ovaries stained with hematoxylin and eosin. The ovaries of *T*♀*D*+ and *T*♀*D*- animals are characterized by increased number of small-sized primordial follicles resembling the manifestation of polycystic ovary syndrome.

3.2.2. Arterial morphology

To investigate the morphological alterations in cerebral arteries, the wall thickness of vessels was determined on hematoxylin- and eosin-stained sections (Figure 13A). Neither VDD nor androgen excess caused any changes in the wall thickness of females (Figure 13B). Surprisingly, however, combined VDD and hyperandrogenism significantly increased the wall thickness (Figure 13B). In accordance with these findings, the lumen cross sectional area was only decreased by combined VDD and androgen excess (Figure 13C), indicating that VDD results in increased active tension and/or inward remodeling in the presence of testosterone.

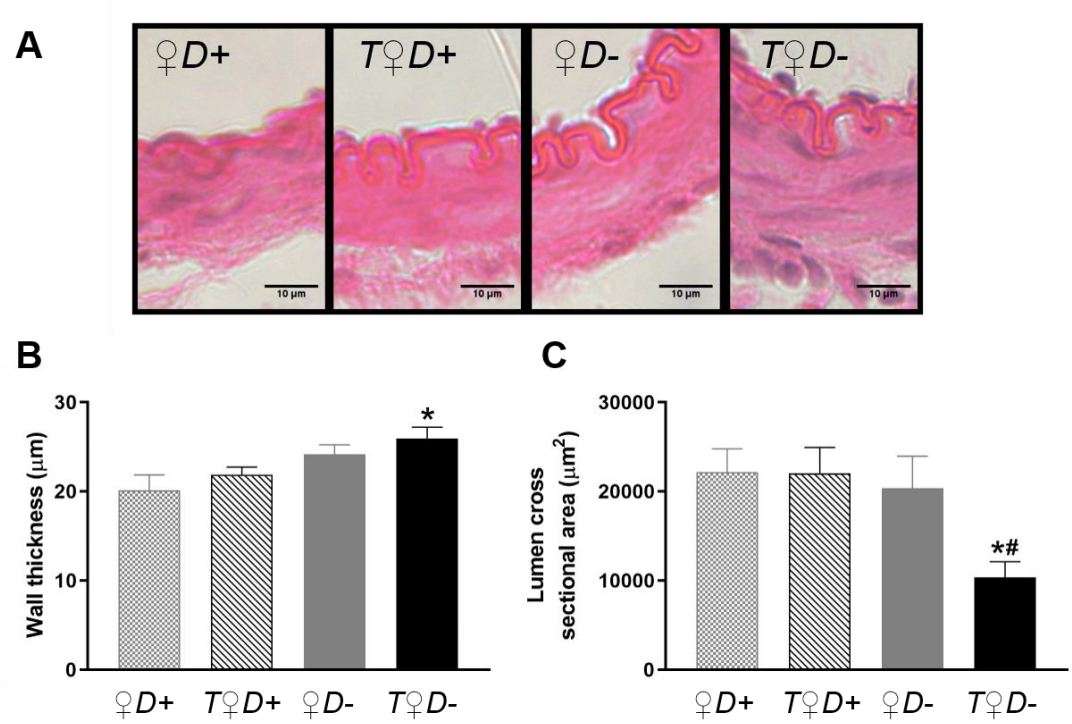

Figure 13. Cerebrovascular remodeling in combined VDD and hyperandrogenism. (A) Representative images of cerebral arteries of female rats stained with hematoxylin and eosin. (B) The combined effect of VDD and androgen excess caused an increase in wall thickness and (C) a decrease in lumen cross sectional area of female rats (*$p<0.05$ vs. ♀*D+*, #$p<0.05$ vs. *T*♀*D+*, two-way ANOVA followed by Tukey's post hoc test, B: n=5-7, C: n=10-11). Data are presented as mean ± SEM.

3.3. Effects of vitamin D receptor deficiency on the cerebrovascular adaptation to unilateral carotid artery occlusion

In order to determine the consequences of impaired vitamin D signaling in the cerebrovascular adaptation to ischemia, we investigated the cerebrocortical blood flow (CoBF) changes following CAO using laser-speckle imaging in male mice carrying a mutant, functionally inactive vitamin D receptor ($VDR^{\Delta/\Delta}$) and in their wild-type (WT) littermates. To identify the alteration(s) of the cerebral vasculature responsible for the diminished adaptation in $VDR^{\Delta/\Delta}$ mice, the morphology of pial collaterals between the anterior and middle cerebral arteries was analyzed.

3.3.1. Physiological parameters of mice

The physiological parameters (heart weight, heart weight/body weight ratio, left ventricle weight, brain weight, heart rate, respiratory rate, plasma ion concentrations) of $VDR^{\Delta/\Delta}$ mice did not differ from those of the WT animals, except for body weight (30.0±0.69 g for WT and 27.3±0.54 g for $VDR^{\Delta/\Delta}$, $p<0.01$, Student's unpaired t-test, n=14-14) and tibial length (1.80 (1.80–1.82) cm for WT and 1.70 (1.60–1.70) cm for $VDR^{\Delta/\Delta}$, $p<0.0001$, Mann-Whitney test, n=14-14). Neither blood pressure (84.09±0.69 mmHg and 82.97±0.54 mmHg for the WT and $VDR^{\Delta/\Delta}$ mice, respectively) nor arterial blood gas parameters (partial pressure of carbon dioxide (pCO_2): 41.16±3.23 mmHg for WT and 42.51±2.19 mmHg for $VDR^{\Delta/\Delta}$; partial pressure of oxygen (pO_2): 93.5 (89.0–103.09) mmHg for WT and 109.0 (85.5–112.8) mmHg for $VDR^{\Delta/\Delta}$; oxygen saturation: 96.10 (94.95–97.58) % for WT and 97.60 (93.40–97.98) % for $VDR^{\Delta/\Delta}$) were impacted by the ablation of VitD signaling. Therefore, we could exclude the possibility that any differences in the systemic arterial blood pressure or in the arterial blood gas tensions would influence the CoBF changes after CAO.

3.3.2. Regional cerebrocortical blood flow changes after carotid artery occlusion

The CoBF was measured using laser-speckle imaging to analyze the changes following CAO. The alterations in CoBF were determined in four different cerebrocortical regions (frontal, parietal, temporal cortices, and the zone of pial anastomoses of both hemispheres) in order to examine the effect of CAO on regional CoBF (Figure 14). Qualitative assessment of the spatiotemporal pattern of CoBF reduction already indicated more pronounced changes in VDR-deficient mice in terms of both the extent and the duration of the hypoperfusion (Figure 14).

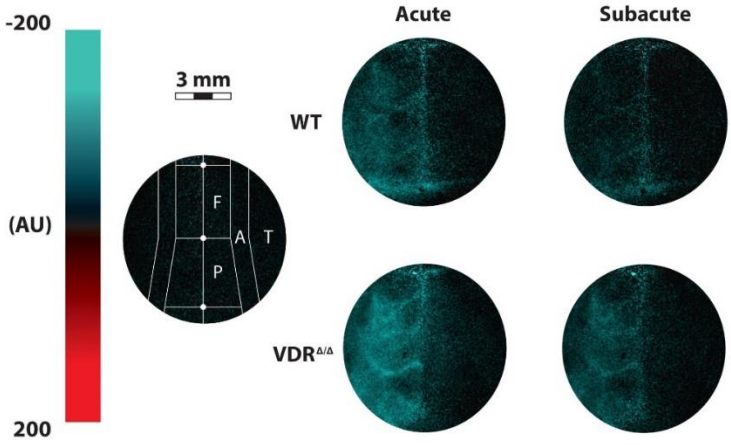

Figure 14. Localization of the regions of interest for cerebrocortical blood flow (CoBF) measurements and CoBF reductions in the acute and subacute phase of adaptation after left carotid artery occlusion (CAO). The decrease in CoBF ipsilateral to CAO was more pronounced in the VDR$^{\Delta/\Delta}$ mice as compared to WT animals with the most sustained reductions in the temporal cortex. The first 30 sec after CAO was considered as the acute phase, whereas the following 270 sec as the subacute phase of adaptation. AU: arbitrary units, F: frontal cortex, P: parietal cortex, A: zone of pial anastomoses, T: temporal cortex

Quantitatively, in the frontal region ipsilateral to CAO neither WT nor VDR$^{\Delta/\Delta}$ mice showed significant CoBF reduction after CAO as compared to the contralateral side (Figure 15A,B), and similar results were obtained in the parietal region of WT animals (Figure 15C). On the contrary, in the parietal region of VDR$^{\Delta/\Delta}$ mice, CoBF was reduced significantly in the acute phase after CAO and normalized thereafter (Figure 15D). More pronounced changes were observed in the temporal region: CAO resulted in pronounced but transient hypoperfusion in the ipsilateral temporal cortex in WT animals, whereas CoBF remained significantly reduced during the whole measurement in VDR$^{\Delta/\Delta}$ mice (Figure 15E,F). In the zone of pial anastomoses, both WT and VDR$^{\Delta/\Delta}$ mice showed transient hypoperfusion in the ipsilateral hemisphere as compared to the contralateral side, although CoBF recovered much later in VDR$^{\Delta/\Delta}$ than WT animals (Figure 15G, H).

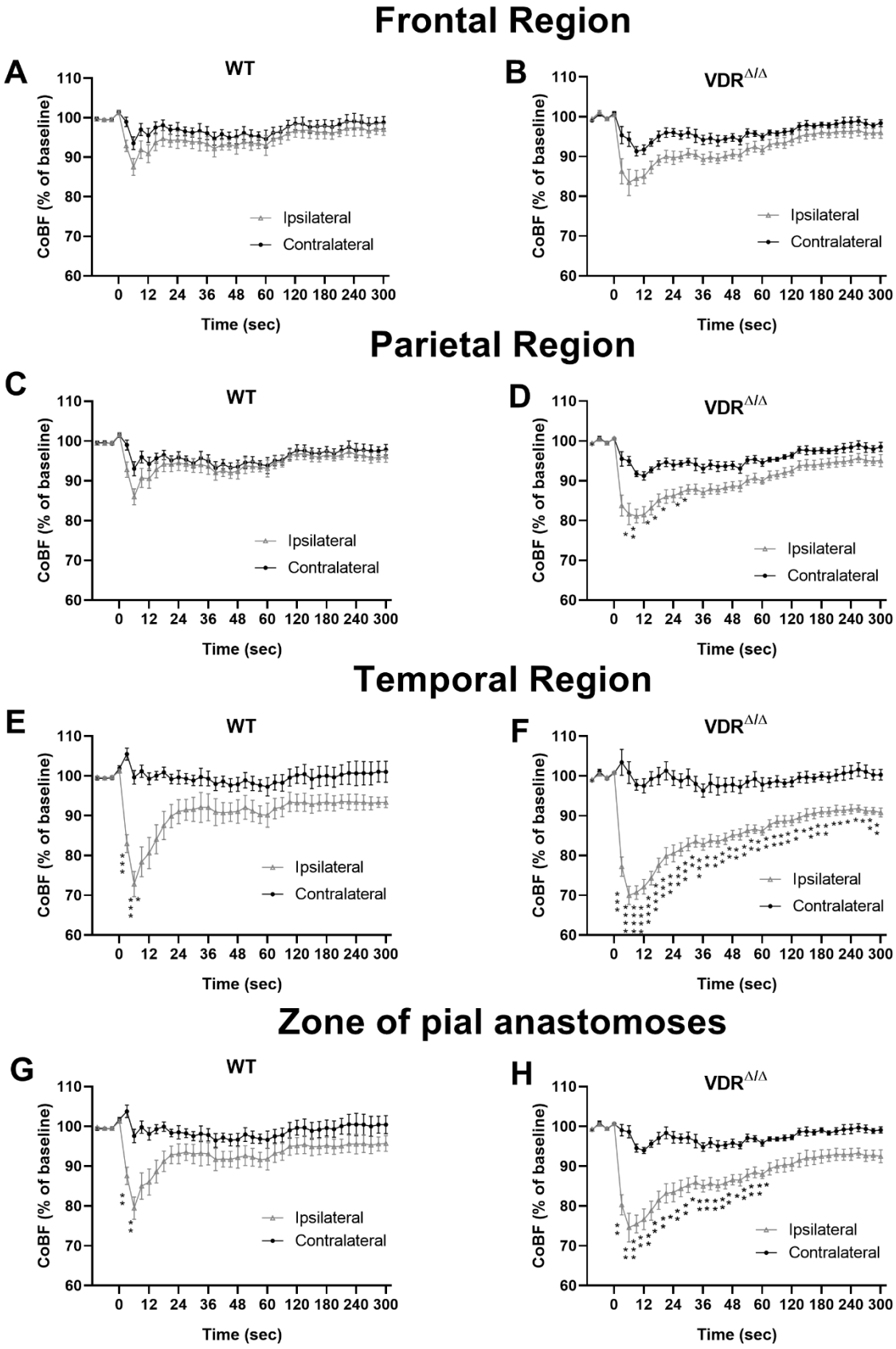
Frontal Region
A
WT
B
VDR$^{\Delta/\Delta}$
Parietal Region
C
WT
D
VDR$^{\Delta/\Delta}$
Temporal Region
E
WT
F
VDR$^{\Delta/\Delta}$
Zone of pial anastomoses
G
WT
H
VDR$^{\Delta/\Delta}$
CoBF (% of baseline)
Time (sec)
Ipsilateral
Contralateral

Figure 15. Regional cerebrocortical blood flow (CoBF) changes in WT (A, C, E, G) and VDR$^{\Delta/\Delta}$ (B, D, F, H) mice following carotid artery occlusion (CAO). Zero indicates the moment of the left carotid artery occlusion. Black circles and gray triangles represent CoBF in the contralateral and ipsilateral hemispheres, respectively. CAO did not cause any changes in the CoBF of the ipsilateral frontal cortex as compared to the contralateral one either in WT (A) or in VDR$^{\Delta/\Delta}$ (B) mice. (C) CAO did not induce any changes in the CoBF of the ipsilateral parietal cortex as compared to the contralateral one in WT mice, whereas (D) it resulted in a significant CoBF reduction in the acute phase in VDR$^{\Delta/\Delta}$ mice. (E, G) The CoBF of the temporal cortex and that of the zone of pial anastomoses ipsilateral to CAO were reduced significantly only in the first few seconds after CAO in WT animals, whereas (F, H) a more prolonged CoBF reduction of both zones was determined in VDR$^{\Delta/\Delta}$ mice. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, two-way repeated measures ANOVA followed by Bonferroni post hoc test) Data are presented as mean ± SEM, n=8 in both groups.

Thereafter, our goal was to quantify the differences in regional CoBF changes induced by CAO in VDR$^{\Delta/\Delta}$ mice as compared to WT animals. In order to pinpoint the direct effect of CAO unmasked by CoBF alterations related to potential fluctuations of systemic physiological parameters (e.g. arterial blood pressure or blood gas values), we determined the area between the CoBF curves (expressed as the percentage of the baseline) of the hemispheres ipsilateral and contralateral to CAO for each mouse. Furthermore, we differentiated between the acute (0-30 sec after CAO) and subacute (30-300 sec after CAO) phases of CoBF changes, as autoregulation may involve different mechanisms with time (84). Functional VDR inactivity caused a more pronounced decrease of CoBF in the parietal region and in the zone of pial anastomoses in the acute phase (Figure 16C,G), indicating an impaired cerebral vasoregulation, whereas during the subacute phase this difference disappeared (Figure 16D,H). In contrast, the CoBF reduction was more pronounced and prolonged in the temporal cortex of VDR$^{\Delta/\Delta}$ mice – as it was increased in both the acute and subacute phases – as compared to the WT animals (Figure 16E, F), indicating a more severe vasoregulatory dysfunction in the temporal cortex of VDR$^{\Delta/\Delta}$ mice.

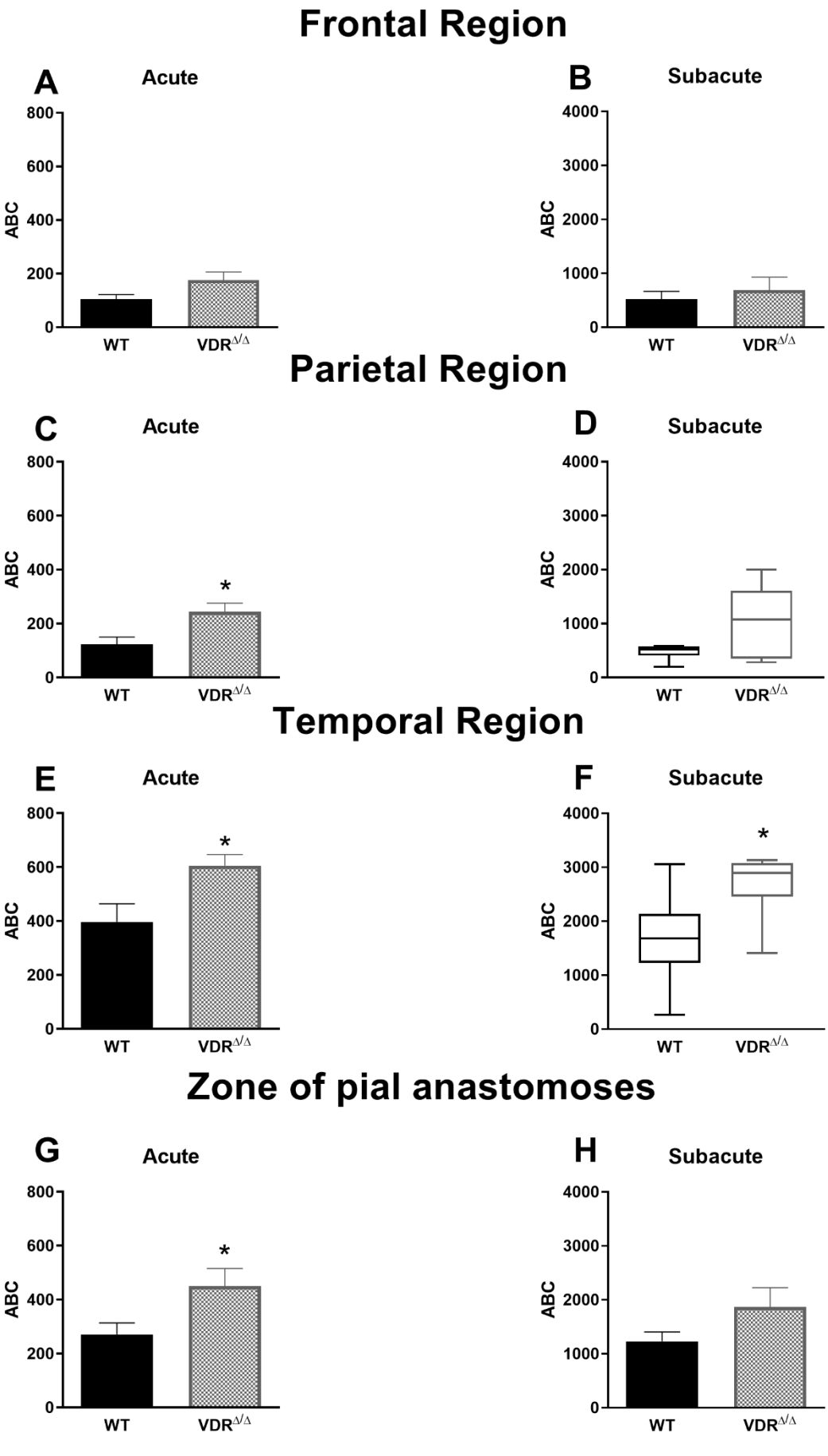
Frontal Region
A
Acute
B
Subacute
Parietal Region
C
Acute
D
Subacute
Temporal Region
E
Acute
F
Subacute
Zone of pial anastomoses
G
Acute
H
Subacute
ABC
WT
VDR$^{\Delta/\Delta}$
*

Figure 16. Differences in regional cerebrocortical blood flow (CoBF) changes induced by carotid artery occlusion (CAO) in $VDR^{\Delta/\Delta}$ vs. WT mice in the acute (0-30 sec after CAO) and subacute (30-300 sec after CAO) phases of adaptation. CoBF reductions induced directly by CAO were determined as the area between the curves (ABC) of CoBF reductions ipsilateral and contralateral to CAO. (A, B) In the frontal cortex, the ABC did not differ between the groups (Student's unpaired t-test). (C) The ABC in the parietal cortex was increased in $VDR^{\Delta/\Delta}$ mice as compared to WT animals in the acute phase (*$p<0.05$, Student's unpaired t-test) indicating a more severe hypoperfusion in the $VDR^{\Delta/\Delta}$ mice; however, (D) this difference disappeared in the subacute phase (Mann-Whitney test). (E, F) VDR inactivity resulted in increased ABC (i.e. decreased CoBF) in the ipsilateral temporal cortex in both the acute (E, *$p<0.05$, Student's unpaired t-test) and subacute phases (F, *$p<0.05$, Mann-Whitney test). (G) ABC was increased in the zone of pial anastomoses of $VDR^{\Delta/\Delta}$ mice as compared to WT animals in the acute phase (*$p<0.05$) but (H) not in the subacute phase (Student's unpaired t-test). Data are presented as mean ± SEM or median and interquartile range, n=8 in both groups.

3.3.3. Effects of VDR deficiency on the intracranial collateral circulation

Pial collateral circulation is one of the major determinants of the efficiency of cerebrovascular adaptation to occlusion of the major cerebral arteries (88). Therefore, we hypothesized that unfavorable alterations of the leptomeningeal anastomoses of the VDR-deficient mice may account for the diminished recovery of CoBF after CAO. In order to test this hypothesis, the number and the tortuosity of collaterals between the cortical branches of the middle cerebral artery (MCA) and ACA were determined (Figure 17A) following visualization of the cerebrocortical vasculature. The ablation of VitD signaling caused a reduction in the number of pial MCA-to-ACA collaterals (Figure 17C) and increased tortuosity of these collateral vessels (Figure 17B,E), indicating impaired development of leptomeningeal anastomoses. In addition, the distance of the anastomotic line from the midline was measured in order to distinguish the cortical territories supplied by the MCA and ACA. Interestingly, the anastomotic line was closer to the midline in $VDR^{\Delta/\Delta}$ mice as compared to WT animals (Figure 17D), indicating that the territory supplied by the MCA was increased at the expense of the territory of ACA. All these alterations have a negative impact on the capacity of leptomeningeal collaterals in the

adaptation of the cerebrocortical circulation to CAO and can explain the more pronounced drop and delayed recovery of CoBF in the temporal cortex of VDR$^{\Delta/\Delta}$ mice.

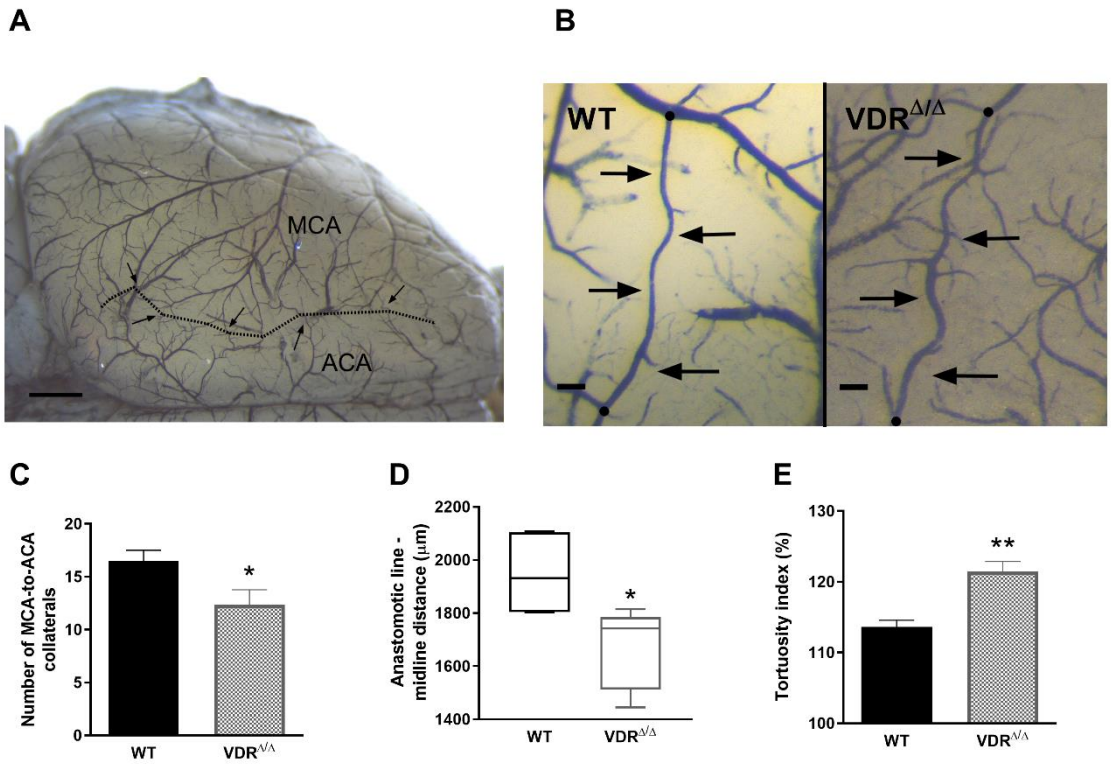

Figure 17. Morphological alterations in the pial collaterals of VDD mice. (A) Representative image of the dorsal surface of the brain infused with the mixture of black inks. The dotted line depicts the anastomotic line, whereas arrows denote the collaterals between the branches of the middle cerebral artery (MCA) and anterior cerebral artery (ACA). Scale bar represents 1000 μm. (B) Representative images indicating the increased tortuosity of the collaterals of VDR$^{\Delta/\Delta}$ mice (right) as compared to WT mice (left). Arrows show the path of one MCA-to-ACA collateral, whereas black dots denote the end points of the collateral. Scale bars represent 100 μm. (C) Functional inactivity of VDR decreased the number of MCA-to-ACA collaterals (*$p<0.05$, Student's unpaired t-test). (D) The anastomotic line was closer to the midline at 4 mm posterior from the frontal pole (level of bregma) in VDR$^{\Delta/\Delta}$ mice as compared to WT animals (*$p<0.05$, Mann–Whitney test). (E) The tortuosity index was increased in VDR$^{\Delta/\Delta}$ mice as compared to WT animals (**$p<0.001$, Student's unpaired t-test). Data are presented as mean ± SEM or median and interquartile range; n=6 in both groups, n indicates the number of brains analyzed.

4. Discussion

Although vitamin D deficiency is associated with increased risk for cerebrovascular diseases (5, 90-94), the role of VitD in cerebrovascular regulation, and thus, in adaptation to cerebral ischemia is still unclear. Additionally, androgen status influences the risk for cerebrovascular disorders and the effects of VDD may also be impacted by gonadal steroids (111, 112, 115), therefore an interplay is assumable between VitD and androgens. In order to elucidate the role of VitD, we examined (i) the VDD-induced morphological and functional alterations of cerebral arteries, and (ii) the role of androgens in the cerebrovascular manifestation of VDD, as well as (iii) the cerebrovascular adaptation to unilateral CAO in mice carrying functionally inactive VDR. Our results indicate that VDD impairs the morphological characteristics and reactivity of cerebral arteries characterized by hypertrophic remodeling, increased vessel tone, endothelial dysfunction, increased COX-2, decreased eNOS as well as AR protein expression. Interestingly, the cerebrovascular manifestation of VDD appears to require higher androgen levels, implying a marked interplay between androgens and VDD in the cerebral circulation. Furthermore, ablation of VitD signaling impedes the rapid recovery of CoBF following CAO, which may be attributed – at least partly – to the impaired development of leptomeningeal collaterals.

VitD influences several pathways relevant to vascular functions, thus VDD appears to be a significant risk factor for cardiovascular and cerebrovascular diseases (4-6). Although observational studies have reported that VDD is linked to several risk factors of stroke such as hypertension, atherosclerosis, dyslipidemia, insulin resistance and diabetes mellitus (6), the causal associations, for instance, between VDD and insulin resistance (132, 133) or between VDD and hypertension (32) are not fully confirmed, especially in young healthy subjects (33, 34, 134). In rodent models, the impact of VDD on blood pressure is controversial: some studies reported that VDD induced hypertension, whereas blood pressure remained unaltered in other studies (32). However, once hypertension developed, it is considered to be the consequence of the VDD-induced upregulation of renin gene expression (135, 136). According to the literature, the development of hypertension appears to depend on the time of onset and the duration of VDD. For instance, VDD during the prenatal period leads to increased blood pressure in rodents' offsprings (64, 75, 137-139). Interestingly, VDD *in utero* contributes to the

development of hypertension in later life even if the offsprings were fed with VitD-sufficient chow after weaning (137, 138), which implies that VDD during pregnancy can severely impact the offspring's long-term health and lead to vulnerability to cardiovascular diseases in adult life (3, 136). However, when VDD affects only the postnatal period of life, the observations are controversial both in juvenile and adult rodents; nevertheless, longer exposure to VDD appears to increase the risk of hypertension (67, 137, 140-145). In our study, weaned rats were exposed to VDD for only 8 weeks, which explains the unaltered blood pressure levels. In addition, VDD has been reported to increase the risk for insulin resistance and diabetes mellitus (108); however, we did not detect any changes in the serum levels of glucose during the oral glucose tolerance test, which indicates that short-term VitD deprivation did not impair glucose homeostasis. Similarly, Hadjadj et al. reported no difference in serum glucose levels between VitD-sufficient and deficient female rats (146). At the same time, VDD appears to lead to insulin resistance in rats (146-148) accompanied with the development of vascular insulin resistance, since VDD appears to diminish insulin-induced relaxation of coronary arteries (146). Taken together, results of basic and clinical studies indicate that VDD may accelerate the development of insulin resistance (149). With this notion, we could not exclude the possibility that longer exposure to VDD would induce insulin resistance in male rats. Since VitD might influence steroidogenesis of sex hormones (150), we determined their concentrations after the 8-week-long VitD deprivation. Although human studies indicate that 25(OH)D levels are associated with serum testosterone concentrations in men (151), the serum levels of sex hormones were not impacted by VDD in our study.

Similarly, ablation of VitD signaling did not cause any major alteration in the measured parameters of the cardiovascular system in male mice. On the contrary, lower body weight and shortened tibial length were observed in VDR-deficient mice, which was in accordance with previous reports (55, 152-154). With regard to the heart weight/body weight ratio, in our study it did not differ between VDR-deficient and WT animals; however, some studies report increased heart weight/body weight ratio in VDR deficiency (55, 155-157). This could be either because older mice were examined or because the animals did not receive the rescue diet for normalizing plasma Ca^{2+} levels given in these studies (55, 152, 155-157). In support of this concept, Andrukhova et al.

found that 9-month-old VDR-ablated mice on rescue diet had increased heart weight/body weight ratio, however, they did not observe this difference in younger animals (55). Similarly, other studies reported increased heart weight/body weight ratio of 12-month-old VDR-deficient mice (155, 156) or younger animals not receiving the rescue diet (157). Consequently, VitD-deprivation or ablated VitD signaling are unlikely to induce any alterations in the systemic cardiovascular parameters (e.g. in blood pressure) in young adult rodents.

Surprisingly, in spite of the well-preserved systemic cardiovascular and metabolic parameters, marked hypertrophic remodeling was observed in the cerebral arteries of VitD-deficient rats, similar to that observed in secondary hypertension (158). In rats and humans suffering from secondary hypertension the increase in the wall-to-lumen ratio of small vessels is due to hypertrophic remodeling as a consequence of smooth muscle cell proliferation (158, 159). In contrast, the hypertrophic remodeling observed in our present study develops at normal blood pressure, therefore it is likely to be the direct effect of VDD on VSMCs. Accordingly, the increased cross-sectional area of the vessel wall appears to be the consequence of VSMC proliferation, because we observed increased number of smooth muscle cells in the tunica media of arteries of the VDD group as compared to controls. While physiological concentrations of $1,25(OH)_2D$ appear to prevent VSMC proliferation (24), the lack of the inhibitory actions in VDD is likely to lead to cell proliferation and subsequent remodeling of the vessel wall. Interestingly, unlike the tunica media, the morphological properties of the tunica intima were not affected by VDD.

Surprisingly, VDD resulted in decreased AR protein expression in the artery wall, of note, despite unaltered androgen hormone levels. Cross-talks between AR- and VDR-mediated gene expressions (124, 125) and the role of VitD in the regulation of AR expression in prostate cells (160) and chondrocytes (161) have been reported previously, implying an interaction between VitD and androgens. Moreover, VitD treatment might increase the expression of AR (160), which is in accordance with our findings that VDD leads to a decrease in AR protein expression. Since endogenous androgen activity *via* AR may protect against vascular remodeling (162) due to increasing the expression of p27 (cyclin-dependent kinase inhibitor), thus, inhibiting VSMC proliferation and migration (163), the decreased AR protein expression might be deleterious in males. Interestingly,

androgens *via* AR have been reported to be an important regulator of the VitD-mediated antiproliferative actions in human prostate cancer cells (160). Taken together, in addition to the attenuated direct effect of VitD on VSMCs, the decreased AR protein expression is likely to contribute to the development of vascular remodeling in VDD. Besides VSMC proliferation, impaired extracellular matrix homeostasis (6) and altered VEGF expression (51, 164) could also contribute to vascular remodeling in VDD. All of these alterations may lead to the development of hypertension, atherosclerosis, thrombosis and arterial stiffness in the long-term (4, 6), which might aggravate the risk of stroke events (81).

In hypertension, increased tangential wall stress facilitates wall thickening to compensate for increased circumferential stress (165). In our study, however, mean arterial blood pressure did not differ between the groups, thus the observed wall thickening resulted in decreased tangential wall stress according to the Laplace equation. Therefore, the lack of the inhibitory effect of VitD on VSMC proliferation (27) may lead to increased wall thickness and consequently to decreased tangential wall stress. The incremental elastic modulus and distensibility did not differ between the groups, indicating that elastic element density and arrangement were not influenced by VDD. This presumption was also confirmed by the unaltered elastic fiber density observed on histological sections. Similarly, no alterations have been found in the elastic fibers of the aorta of 3-month-old VDR deficient mice; however, the elastin protein content was decreased at the age of nine months (55). Therefore, we could not exclude the possibility that longer exposure to VDD may cause alterations in elastic fiber density and thus in the arterial stiffness of cerebral arteries. Taken together, 8-week-long VDD appears to impair vascular morphology characterized by hypertrophic remodeling, but it is unlikely to impair the elastic properties of the vessel wall.

VDD has been associated with endothelial dysfunction (20), therefore we examined the endothelial relaxation capacity following the application of bradykinin. Bradykinin is known to relax cerebral arteries *via* the B_2 receptor and NO release (166); however, in the presence of endothelial dysfunction, bradykinin causes endothelium-independent contractions (167). In our experiment, bradykinin failed to relax the arteries of the VDD group, indicating the development of endothelial dysfunction. VitD has been reported to stimulate NO production through eNOS activation (61) or due to an increase in eNOS expression (59), as detailed previously. Accordingly, we observed decreased eNOS

protein expression in the endothelium of VitD-deficient rats, which could impair the endothelium-dependent relaxation capacity of arteries. In the case of endothelial dysfunction, bradykinin-induced contractions might be mediated by COX-2-derived prostanoids and activation of TP receptors (167), therefore the increased COX-2 expression might also contribute to the lack of vasodilation in VDD. As VitD has been reported to downregulate the expression of COX-2 (70), it may be a modulator of the prostanoid system. Additionally, VitD is likely to downregulate the expression of TP receptors (70), which in turn leads to diminished NO production, since activation of TP receptors has been reported to decrease eNOS activity (168). Therefore, in VDD, the increased signaling *via* TP receptors may contribute to the impaired NO-mediated vasodilation. Since VDD is associated with enhanced production of pro-inflammatory mediators and ROS (6), the endothelium-dependent vasodilatation is also likely to be diminished by increased oxidative stress. ROS, for instance, might cause eNOS uncoupling or inactivation of NO (68), thus its increased production may lead to endothelial dysfunction. Furthermore, impaired AR-mediated signaling due to the decreased AR protein expression in VDD might also contribute to endothelial dysfunction. The role of physiological androgen status in preserving vascular function of men is known (162, 163). Therefore, impaired androgen signaling in cerebral arteries might be deleterious, since decreased levels of eNOS expression and phosphorylation, thus diminished NO bioavailability were observed in the aorta of AR knockout mice (162).

Cerebral arteries possess intrinsic myogenic tone (169), which can increase inappropriately under pathophysiological conditions. In our study, the arteries of VitD-deficient animals developed greater myogenic tone, which is similar to the observation of Tare et al., who reported a twofold enhancement of the myogenic tone of mesenteric arteries in male VitD-deficient rats as compared to VitD-sufficient ones (64). In addition, UTP – a potent and partly thromboxane A_2-mediated constrictor of cerebral arteries (170) – induced greater tone in the ACA of VDD animals. Therefore, VDD is likely to increase vascular tone, which may be attributed – at least partly – to increased COX-2 protein expression or TP receptor upregulation. In VDD, the prostanoid balance could be shifted towards vasoconstriction, for instance, due to the inactivation of prostacyclin synthase by ROS (171). Therefore, the enhanced COX-2 expression in VDD might lead to increased

production of vasoconstrictor prostanoids and subsequently to enhanced vascular tone and contractility. Taken together, enhanced vasoconstrictor prostanoid release and sensitivity together with the impairment of the counterbalancing NO pathway could lead to increased myogenic tone and constrictor responses as well as endothelial dysfunction in VDD. Therefore, in addition to the induction of vascular remodeling, VDD may aggravate the risk for stroke due to impairing cerebrovascular functions.

Interestingly, marked changes of vascular morphology and reactivity developed in healthy young adult, male animals within a relatively short period (8 weeks) of VDD. These results indicate that VitD indeed contributes to the preservation of normal cerebrovascular function and, in turn, VDD may increase the risk for cerebrovascular disorders. At the same time, the incidence and severity of cerebrovascular diseases appear to depend on gender (5, 112), and to be influenced by sex steroids (112). Particularly, men and hyperandrogenic women have been reported to be at increased risk for vascular disorders as compared to premenopausal healthy women (111, 112). Hyperandrogenic disorders, for instance PCOS, are associated with morphological alterations of vessels, especially with increased intima-media thickness of carotid arteries, and they are likely to lead to endothelial dysfunction (114). Furthermore, chronic testosterone treatment has been reported to increase the cerebrovascular tone of male orchiectomized rats possibly due to suppressing the activity of large conductance calcium-activated potassium channels, hampering an endothelium-derived hyperpolarizing factor-like vasodilator, and upregulating the thromboxane synthase (112). Although VDD and androgen excess are definitely associated with the risk for cerebrovascular disorders (5, 24, 112, 172), their combined effects on cerebral arteries have not been revealed previously.

To gain further insight into the cerebrovascular impacts of combined VDD and androgen excess, we examined their effects on cerebral arteries of female rats. Surprisingly, neither VDD nor androgen excess alone caused alterations in the vessel lumen size and wall thickness of female rats. On the contrary, some studies report that androgen excess leads to alterations in the diameter or wall thickness of peripheral vessels (173, 174). VDD may impact the morphology and reactivity of arteries of female rats: it causes an increase in myogenic tone and a decrease in endothelium-dependent vasodilation of mesenteric arteries (64), and leads to vascular remodeling as well as alterations in contractility and relaxation ability of coronary arterioles (41). Therefore,

the vascular impact of hyperandrogenism and VDD may depend on vessel type. Importantly, neither disorder alone caused remodeling in the cerebral arteries of females, at least not within 8 weeks. Combined androgen excess and VDD, however, resulted in vascular remodeling of cerebral arteries. Although VDD and hyperandrogenism are likely to be associated with hypertension (6, 175), neither treatment impacted the blood pressure of animals in our study. Therefore, we could exclude the possibility that the observed vascular changes would be secondary consequences of VDD/hyperandrogenism-induced hypertension.

Accordingly, androgen excess appears to be required for the early cerebrovascular manifestation of VDD. Thus, VDD and androgen excess synergistically may cause deleterious alterations in the morphology and reactivity of the ACA within a relatively short time, which could facilitate the development of cerebrovascular diseases or aggravate their outcomes. This finding indicates an interaction between VitD and androgens in the cerebral circulation. Furthermore, our results might imply that post-menopausal and hyperandrogenic women are at increased risk of the cerebrovascular consequences of VDD.

Interestingly, VDD appears to induce similar alterations in hyperandrogenic females and in males, however the mechanism might depend on gender. While VDD causes vascular remodeling (Figure 4-7 and 13) and decreases endothelium-dependent relaxation (Figure 11A and (176)) in the cerebral arteries of both males and hyperandrogenic females, the VDD-induced enhancement of cerebrovascular contractility was only present in males (Figure 11C), but not in hyperandrogenic females (176). These results support the findings that (i) short-term VDD alone does not cause any alterations in cerebral vessels of females, and (ii) men are more seriously affected by the cerebrovascular consequences of VDD than healthy premenopausal women. Importantly, men with combined VitD and androgen deficiency are at high risk for total and cardiovascular mortality (177), suggesting that physiological androgen and VitD levels are a prerequisite for their health.

Our results indicate that short-term VDD – at least in males – can lead to remodeling and impaired function of cerebral arteries. These findings imply that VDD may impair the cerebral blood flow regulation and in turn the cerebrovascular adaptation to ischemia. Occlusion of a major artery supplying the brain such as the carotid artery could

compromise the cerebral circulation and cause hypoperfusion of the brain tissue (83). At the same time, compensatory mechanisms are activated in order to maintain cerebral blood perfusion. For instance, unilateral CAO has been shown to induce rapid reduction in the CoBF of the ipsilateral hemisphere in mice, but within 30 sec, the CoBF starts to increase and returns close to the baseline level (84). However, ablation of VitD signaling appears to impede the rapid recovery of CoBF following CAO, since we determined more pronounced CoBF reductions in the ipsilateral hemisphere of $VDR^{\Delta/\Delta}$ mice as compared to WT animals.

Following unilateral CAO, the intracranial collateral circulation (large vessels of the Willis circle and smaller pial anastomoses between the terminal branches of the anterior, middle and posterior cerebral arteries) supplied primarily by the contralateral carotid artery represents the first line of defense against ischemia (84, 85, 88). In mice, the ACAs of the two sides fuse and give rise to the azygous anterior cerebral artery (AACA), which supplies the frontoparietal regions of both hemispheres (178). Consequently, efficient blood supply from the contralateral side provided due to the AACA is likely to protect the frontal-parietal cortex from unilateral CAO (84, 178). Despite the lower vulnerability of these regions, the CoBF decreased significantly and remained reduced in the acute phase (i.e. until 30 sec after CAO) in the parietal cortex in $VDR^{\Delta/\Delta}$ mice. We presume that the large vessels of the Willis circle are impaired in VDR inactivity, which could impede the recovery of the parietal cortex following CAO. In support of this concept, we found that VDD caused morphological and functional alterations in ACA of rats. For instance, VDD diminished the protein expression of eNOS in the arterial wall (Figure 10) implying decreased NO production, which in turn impaired the endothelium-dependent relaxation (Figure 11). Taken together, the compromised flow-induced vasodilatory capacity of the vessels of the Willis circle could be responsible for the insufficient blood flow redistribution in the acute phase (0–30 sec after CAO) and in turn for the lack of immediate adaptation to CAO in VDR deficiency.

Although the temporal cortex is more severely affected by CAO (84), the pial collateral circulation can attenuate its ischemia (84, 89, 179) through blood flow redistribution between the more severely affected temporal cortex and the less severely impacted frontal and parietal regions after CAO (84, 180). In these anastomoses, the blood can flow in both directions depending on the hemodynamic status and metabolic

needs of the connected territories, therefore they can improve the blood supply of the more ischemic region (87). Our results indicate that this compensatory mechanism could not work sufficiently in the absence of VitD signaling, because CoBF reduction in the temporal cortex was more severe and prolonged, i.e. the recovery was slower in $VDR^{\Delta/\Delta}$ mice as compared to WT animals. Since VitD might modulate vasculogenesis (50, 54), the development of leptomeningeal collaterals is likely to be influenced by VitD signaling. Therefore, we examined the pial collaterals between the cortical branches of the MCA and the ACA to evaluate the capacity of the intracranial collateral circulation to compensate for the blood loss of the temporal cortex in VDR deficiency. The abundance of leptomeningeal anastomoses among the terminal branches of the three large cerebral arteries is the highest between the MCA and the ACA, and these collaterals might be particularly important for the blood flow redistribution between the more severely affected temporal cortex and the less severely impacted frontal and parietal regions after CAO (84, 180). Importantly, the extent of the pial collateral network appears to be inversely associated with the cortical infarct size (89, 181), therefore, the decreased number of anastomoses in $VDR^{\Delta/\Delta}$ mice could exacerbate the consequences of ischemic stroke. The decreased collateral number was accompanied by increased tortuosity of anastomoses in $VDR^{\Delta/\Delta}$ mice, which further compromises the collateral circulation. The increased vascular tortuosity implies the development of local turbulence and may cause abnormal shear stress in the vessel wall, resulting in impaired flow-induced vasodilation, which may ultimately lead to the generation of atherosclerosis (182).

In addition to the differences in collateral density and morphology, the size of the MCA and ACA tree (i.e. the cerebral territory supplied by the MCA and the ACA) impacts the outcome of stroke (183). Functional inactivation of VDR increased the territory of the MCA at the expense of that of the ACA, which might further impair the compensatory capacity, since a larger territory has to be supplied by the less developed collateral network. Furthermore, collateral vessel diameter could also impact the blood flow redistribution capacity of anastomoses (184, 185) which is likely to be influenced by VitD signaling, since we found that VDD leads to vascular remodeling in cerebral arteries. Nevertheless, the impact of VitD signaling on collateral diameter is yet to be investigated.

Pial collaterals of mice begin to form at around the prenatal day 15 and achieves the adult density by postnatal day 21 (186). Therefore, VDD, especially in the prenatal or perinatal period (186) may lead to impaired pial collateral circulation. The development of collaterals might be compromised, for instance, by diminished VEGF expression during the embryogenic life (38, 186, 187). Since VitD induces the upregulation of VEGF (17, 42, 50, 51), the reduced VEGF expression might be attributed to the impaired collateral development in VDR deficiency. Furthermore, NO may impact the maintenance of collateral circulation, as a significant decrease in collateral number was observed during growth to adulthood in eNOS knockout mice (188). Thus, the decreased eNOS expression in the cerebral arterial wall and, subsequently, the diminished NO production may also contribute to the reduced collateral density in VDR deficiency. Taken together, our results indicate that VitD plays an important role in collateral development, and in turn, the absence of VitD, especially in the prenatal or perinatal period may lead to impaired pial collateral circulation. The latter might hamper the draining effect between the temporal and frontal-parietal region in the subacute phase (30-300 sec after CAO) and consequently could be responsible for the prolonged CoBF reduction in the temporal cortex. Importantly, the more severe and prolonged hypoperfusion of $VDR^{\Delta/\Delta}$ mice is unlikely to result from any changes in systemic arterial blood pressure or blood gas parameters, because we did not detect any differences in those parameters between the experimental groups.

The VDD-induced morphological and functional changes of the large vessels of the Willis circle and smaller pial anastomoses may diminish the capacity of the cerebral circulation to rapidly respond to occlusion of a cerebral artery. Therefore, VDD compromises the cerebrovascular adaptation to ischemia and worsens the outcome of ischemic stroke. With this notion, VDD has been reported to increase the infarction volume, exacerbate behavioral impairment, and compromise the blood–brain barrier after cerebrovascular occlusion (103, 104). However, Evans et al. found that VDD had no effect on the extent of brain injury (107). The discrepancy could be explained by the different onset and type of VDD. In our study, mice carrying a functionally inactive VDR were exposed to the absence of vitamin D signaling already in the prenatal period, which appears to lead to more severe vulnerability to cardiovascular diseases than VDD developing in adult life (3, 136). Accordingly, developmental VDD has been reported to

alter neuronal growth and differentiation, and it is associated with impaired development of the dopaminergic system (189) as well as with the risk of schizophrenia (190, 191). Furthermore, VDD appears to be linked to autism, Parkinson's disease, cognitive impairments, and depression (190, 191). The beneficial role of VitD in the brain could be attributed – at least partly – to its neuroprotective effect (19), since VitD has been reported to modulate the expression of neurotrophic factors, ion channels, and inflammatory mediators (189). Therefore, besides compromising cerebrovascular adaptation, VDD can also worsen the outcome of ischemic stroke by directly impairing neuronal functions.

In conclusion, our results indicate that VDD induces deleterious alterations in cerebral vessels, which in turn impair the cerebrovascular adaptation to ischemia. Therefore, VDD increases the risk and worsens the outcome of stroke. However, some questions remain to be answered. For instance, how do the time of onset and duration of VDD influence its cerebrovascular consequences? Are the cerebrovascular consequences of VDD reversible? Furthermore, what is the exact mechanism underlying the gender (androgen)-dependence of the cerebrovascular manifestation of VDD? Although we observed that VDR deficiency impaired the cerebrovascular adaptation to CAO in male mice, we could not exclude the possibility that we would not see the same effects in female mice. Our results in accordance with other animal and human studies (4-6, 103, 104) imply that VDD favors the development and worsens the outcome of cerebrovascular disorders; however, large Mendelian randomization studies have failed to provide evidence for causal association between 25(OH)D levels and ischemic stroke in humans (100-102). Nevertheless, our results strongly support the view that physiological VitD status plays a major role in the development and functions of cerebral vessels and in preventing their disorders.

5. Conclusions

In our experiments, we aimed to investigate the impacts of vitamin D deficiency on the morphological and functional characteristics of cerebral circulation. Our results indicate that:

- Vitamin D deficiency induces hypertrophic remodeling due to enhanced vascular smooth muscle cell proliferation in cerebral arteries of male rats.
- Vitamin D deficiency causes an increase in vessel tone and a decrease in endothelial relaxation capacity accompanied by enhanced COX-2 and decreased eNOS and AR protein expression in cerebral arteries.
- In female rats, vitamin D deficiency combined with androgen excess leads to vascular remodeling in cerebral arteries. Cerebrovascular manifestation of vitamin D deficiency requires androgen excess and depends on gender, implying an interplay between androgens and vitamin D in the cerebral circulation.
- Ablation of vitamin D signaling compromises the cerebrovascular adaptation to unilateral carotid artery occlusion in male mice characterized by reduced cerebrocortical blood flow in the parietal and temporal regions of the ipsilateral hemisphere.
- The temporal cortex of vitamin D receptor deficient mice shows the most pronounced drop and delayed recovery following carotid artery occlusion, which could be attributed to the impaired development of leptomeningeal anastomoses characterized by decreased number, increased tortuosity and altered location of collaterals between the middle and anterior cerebral arteries.

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6. Summary

Vitamin D (VitD), which is a lipid-soluble vitamin that functions as a steroid hormone, plays an important role in many physiological processes including cellular proliferation, differentiation, angiogenesis, oxidative stress, and immune functions. Accordingly, vitamin D deficiency (VDD) has been linked to several disorders including cerebrovascular diseases. Although VDD has been associated with increased risk and severity of ischemic stroke, the impact of VitD on cerebral arteries is uncertain. Therefore, we aimed to analyze the effects of VDD on the morphological, biomechanical and functional properties of cerebral arteries. We observed that VDD induces hypertrophic remodeling resulting from enhanced vascular smooth muscle cell proliferation, and increased vessel tone as well as endothelial dysfunction due to enhanced vasoconstrictor prostanoid production, decreased NO bioavailability and androgen receptor protein expression. Since men and hyperandrogenic women are at increased risk of stroke as compared to premenopausal healthy women, we presumed that androgens influence the cerebrovascular manifestation of VDD. To test this hypothesis, we examined the combined impact of VDD and androgen excess on cerebral arteries of female rats. Interestingly, unlike in males, VDD alone did not cause any alterations in the morphology of cerebral arteries of females. However, combined VDD and androgen excess leads to vascular remodeling in females. Therefore, we assume that the cerebrovascular manifestation of VDD requires androgens and thus, it depends on gender, which implies an interplay between androgens and VitD in the cerebral circulation. Since the VDD-induced changes of cerebral arteries may compromise the cerebral circulation, we examined whether ablation of VitD signaling impacts cerebrovascular adaptation to unilateral carotid artery occlusion, a common consequence of atherosclerosis and cause of ischemic stroke. The cerebrocortical blood flow showed a significantly increased drop and delayed recovery after carotid artery occlusion in VitD receptor deficient mice with the most sustained difference in the temporal cortex. The latter may be attributed to the impaired development of leptomeningeal anastomoses in VitD receptor deficient mice. These results indicate that VitD receptor deficiency compromises the cerebrovascular adaptation to ischemia, and therefore increases the risk and severity of cerebrovascular diseases. In conclusion, physiological VitD status appears to play a major role in the development and function of cerebral vessels and in preventing their disorders.

7. References

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8. Bibliography of the candidate's publications

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