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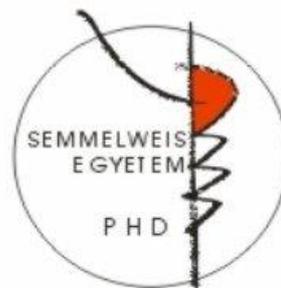
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Effects of vitamin D deficiency and supplementation on coronary and carotid arteries in a rodent model

PhD thesis

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LIST OF ABBREVIATIONS

25(OH)D	25-hydroxy-vitamin D
ACA	Anterior cerebral artery
ANOVA	Analysis of variance
AR	Androgen receptor
CAD	Coronary artery disease
COX	Cyclooxygenase
CV	Cardiovascular
CVD	Cardiovascular disease
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin
FVD	Female vitamin D deficient
HDL	High-density lipoprotein
INDO	Indomethacin
IU	International unit
LAD	Left anterior descending artery
LDL	Low-density lipoprotein
L-NAME	N(G)-Nitro-L-arginine methyl ester
MACE	Major adverse cardiovascular event
MMP	Tissue matrix metalloproteinase
MMP-2	Tissue matrix metalloproteinase-2
MVD	Male vitamin D deficient
NON-STEMI	Non-ST elevation myocardial infarction
NT	3-Nitrotyrosine
NO	Nitric-oxid
PCOS	Polycystic ovarian syndrome
PGI2	prostacyclin
PTH	Parathormone
RAAS	Renin-Angiotensin-Aldosterone System

SHR	Spontaneous hypertensive rat
SMA	Smooth muscle actin
TP	Thromboxane receptor
TNF- α	Tumor necrosis factor - α
ULDL	Ultra low-density lipoprotein
VD	Vitamin D
VDD	Vitamin D deficiency
VDR	Vitamin D receptor
VDRE	Vitamin D Response Elements
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1. Brief overview of vitamin D

The name “vitamin D” has been known and used since 1922, according to McCollum’s work (1). In these 100 years, we have discovered and learned extraordinary things about vitamin D. It is primarily a regulator molecule of the bone metabolism, and calcium homeostasis (2-5). However, over the years, in addition to the systemic effects of skeletal muscle, its prominent role in the development of many diseases and in the normal functioning of the physiological processes has been demonstrated. Suffice it to mention mental problems (6), its role in regulating the immune system (7), or the importance observed in pregnancies and their outcome (8). Taking all this into account, some consider it a complex molecule that influences the physiological processes of the body. To support this, its receptor (vitamin D receptor, VDR) has been found in many tissues of many organs (9-11).

1.2. Vitamin D physiology and mechanisms of action

Vitamin D is primarily a steroid hormone-like molecule that regulates calcium and phosphate metabolism. Natural vitamin D cholecalciferol (D3) is produced in the body in the skin from 7-dehydrocholesterol under the influence of UV light. In addition to its physiological production detected in the skin, cholecalciferol can also be taken with diet or in the form of medicine. Cholecalciferol taken orally does not inhibit the skin's vitamin D3 synthesis. Two hydroxylations are required for the vitamin already present in the body to become active. During this process, cholecalciferol is converted to 1,25 (OH) D3 in two hydroxylation steps. First in the liver (position 25) and then in the kidney in tissue (position 1). The main site of excretion of the active hormone is the bile, and to a lesser extent the kidney. Vitamin D3 and its hydroxy derivatives exert their primary effect in the small intestine, bones, kidneys, and parathyroid glands, regulating the body's calcium, phosphate, and bone metabolism. Detailing these effects, vitamin D and its derivatives increase the absorption of calcium and phosphate from the intestine, raise serum calcium and phosphate concentrations and reduce the excretion of phosphate in the urine. In

interaction with parathormone (PTH), they regulate the calcium and phosphate content of the bones, stimulate the incorporation of calcium into the osteoid and the release of calcium from the bone tissue. As a result of all these effects, vitamin D improves the use of calcium and promotes bone building. It directly affects the recruitment, maturation and activity of osteoblast and osteoclast cells (2-5). The biological activity of 1,25-dihydroxycholecalciferol is mostly mediated by the intracellular vitamin D receptor (VDR), which functions as a ligand-activated transcription factor. The vitamin D receptor belongs to the steroid-thyroid-retinoid receptor superfamily. VDR can be found in many tissues (11). Through the VDR, vitamin D can exert its non-skeletal genomic effects. It has an antiproliferative and differentiation-stimulating effect on fibroblasts (12), hair follicles (13) and other cells, as well as on smooth muscle cells, myoblasts and cardiac muscle cells (14), inhibits the synthesis of natriuretic factor in atrial myocytes (15), increases the production and secretion of insulin in the beta cells of the pancreas (16-20); controls catecholamine metabolism in medullary cells of the adrenal gland (21) and it has an antiproliferative effect on the myometrium and endometrium (22). It enhances lung maturation, phospholipid synthesis and the release of surfactants (23), stimulates the function of Sertoli cells and spermatogenesis (24), and may control the hormone production of the pituitary gland (25). 1,25-dihydroxycholecalciferol also has rapid effects, which are not exerted through changes in gene expression, but are mediated through cell surface receptors. These effects of 1,25-dihydroxycholecalciferol include rapid effects on phosphoinositide metabolism, elevation of intracellular calcium levels, enhancement of intestinal calcium transport and phosphate influx, elevation of cGMP levels, and activation of protein kinase C (26, 27).

1.3. Overview of regulation of the tone of a blood vessel

The spontaneous tension of the muscle elements of the vessel wall is called myogenic tone (28). The endothelium plays an important role in regulating vascular smooth muscle tone, as well as in modulating the adhesion of cellular elements to the vascular wall (29). In response to various effects, endothelial factors are released in the endothelium, which cause a change in vascular smooth muscle tone. The three most important substances of

endothelial origin are: nitric oxide (NO), endothelin (ET-1) and prostacyclin (PGI₂). NO and PGI₂ act as vasodilators, while ET-1 acts as a vasoconstrictor (30-32).

The primary physiological effects of PGI₂, leukotrienes and thromboxanes are related to inflammation, but they are all vasoactive paracrine hormones by nature. As we have seen, PGI₂ is a vasodilator, while other arachidonic acid metabolites, e.g., thromboxane, act as vasoconstrictors. Arachidonic acid is metabolized in cells in two ways. In the cyclooxygenase pathway, which leads to prostaglandins, and in the lipoxygenase pathway, which results in eicosanoid-leukotriene production. The balance of arachidonic acid derivatives formed in endothelial cells, thus the base myogenic tone, may be characteristic of the given vessel and may also show gender differences. Blocking the formation of these metabolites can be a therapeutic goal, and with the experimental use of cyclooxygenase (COX) inhibitors, we can get closer to the discovery of vasoconstrictor and vasodilator mechanisms.

Gender specific alterations of the plasma level of sexual steroids is also an important regulatory factor of the myogenic tone. Female gender seems to result in a decreased spontaneous vascular tone, decreased contractile capacity and increased relaxation (33-35). Vasoconstriction and dilatation differences in different sexes have already been described for many blood vessels. Mesenteric artery contraction induced by vasoconstrictor prostanoids is more pronounced in male hypertensive rats (36). The coronary arteries of male rats have an increased basal tone, the contraction induced by vasoconstrictor prostanoids, and endothelin-1 is stronger than the arteries of female animals (37, 38). Serotonin-induced contraction of carotid arteries is enhanced in male mice (39). Gender differences can be influenced by various diseases and age. The endothelium-dependent dilatation of the brachial arteries of young women with kidney disease is higher than that of men. This difference disappears as time progresses (40).

1.3.1. Vascular tone and vitamin D

Vitamin D acts on cells through two different mechanisms. During the genomic effect, the ligand-binding vitamin D receptor binds to the special promoter regions of more than 200 genes (VDRE -Vitamin D Response Elements) regulated by vitamin D in the nucleus. The so-called non-genomic effect is produced by vitamin D, via receptors located in the cytoplasm and cell membrane of cells, via the calcium signaling pathway. The most

important effect of vitamin D on endothelial cells is the increased production and increased activity of endothelial NO synthase (eNOS). Genetic effects of vitamin D promote eNOS expression via nuclear VDR. As a non-genomic effect, the increase in the intracellular calcium level activates eNOS. As a result of the increasing NO level in the endothelium, angiogenesis increases by increasing the matrix metalloproteinase 2 (MMP-2) gene expression, which improves the migration and proliferation capacity of endothelial cells. Smooth muscle cells react with vasodilation through the increase in endothelial NO. Vitamin D also has a direct effect on smooth muscle cells, like the effect that causes endothelial proliferation, high-dose (toxic overdose, reached by 78.000 IU/kg rat chow) vitamin D treatment increases smooth muscle proliferation. Interestingly, these effects can also be observed in the absence of vitamin D. The result in both cases is inward hypertrophic remodeling, i.e., the wall thickness, wall lumen ratio and wall cross-section also increase (41-47).

1.4. Coronary vascular structure and physiology

The coronary arteries provide the blood supply to the heart's own tissues. The formation of the coronary vessels begins at the time of the division of the fetal aortic trunk into the aorta and pulmonary artery, in the 5th embryonic week. Endothelial precursor elements located around the aortic wall assemble into primitive tubules. In this process, increased metabolism and hypoxia induced by the thickening of the compact myocardium are attributed a role (48). In the process, vascular endothelial growth factor (VEGF), and Platelet Derived Growth Factor (PDGF) levels play a role. The forming tubule network emerges into the aortic wall, where it establishes a connection with the lumen of the aorta during the process of apoptosis. Later, the vasculogenesis that continues in the heart muscle forms the coronary network. The mature, developed coronary artery belongs to the group of elastic arteries. It has a thin tunica adventitia and a thick tunica media containing large amounts of elastic and collagen fibers (49). In addition to the myogenic tone of the vessel wall and the factors that regulate it, the location of these vessels plays an important role in the formation of the blood flow of the coronary network. The blood vessels running on the surface of the heart follow the flow patterns characteristic of arteries, defined by systole and diastole. However, the subendocardial branches are

compressed by the contracting myocardium during ventricular systole, so retrograde flow develops in them (50). So the heart muscle got its blood supplementation mainly during myocardial diastole (51).

1.5. Oxidative nitrative stress and vitamin D

Oxidative stress is the condition when the balance of free radicals and antioxidants in the body is disrupted. The increase of reactive oxygen radicals can lead to the oxidation of proteins and fats, to angiogenesis and pathological angiogenesis through the activation of VEGF (52, 53). Oxygen forms peroxynitrite with NO, which also leads to protein damage. Peroxynitrite also plays a role in tyrosine nitration, so the amount of nitrotyrosine is a marker of oxidative damage to the cell.

Vitamin D has been shown to play a role in mitigating the harmful effects of this oxidative stress. Its effect is clear in the pathophysiology of diabetes (54) in muscle physiology (55) or, for example, in preeclampsia (56). Vitamin D is extremely important for the health of the endothelium from this aspect as well (57). In addition to increasing the release of nitric oxide, vitamin D increases the expression of enzymes that eliminate free radicals, such as superoxide dismutase, glutathione peroxidase and reduces the expression of interleukin-1 and Tumor Necrosis Factor α (TNF- α) (58-61).

1.6. Vitamin D deficiency and cardiovascular health

Serum vitamin D levels in humans are for vitamin D deficiency based on literature data are the follows: (25-hydroxy-vitamin D / 25(OH)D < 20 ng/mL = <50 nmol/L (62, 63). A significant portion of the world's population is affected by vitamin D deficiency. There are, of course, geographical differences, presumably due to differences in the number of hours of sunshine, but it should be noted that it is also prominent in developed countries even in such sunny ones as Australia (64). With the advancement of the Western way of life, it is expected to rise further. However, lifestyle changes are more effective in preventing this than vitamin D supplementation itself (65).

The role of vitamin D in the regulation and processes of the cardiovascular system is prominent. Decreased serum vitamin D levels are clearly associated with cardiovascular mortality (66, 67). We should also mention that the role of vitamin D supplementation in reducing these risks is doubtful based on the literature data. Based on the results of comparative studies, it is unclear whether vitamin D supplementation can reduce this negative cardiovascular effect (9, 67-73), and only a few studies demonstrate clear positive effects of supplementation (74-76). This can be observed in other diseases as well, a typical example of this is that adequate vitamin D levels have a protective effect in COVID-19 infection, but vitamin D supplementation during illness has no clear effect on the outcome of the disease (77). The reason for the apparent contradiction may be the special nature of human studies. In the case of human studies, it is difficult to create homogeneous groups where the vitamin D levels do not show a large deviation, it is known for sure how long the vitamin D deficiency has existed, and whether the vitamin D level was constant during its existence. It is difficult to control the consumption of foods containing vitamin D during the test, as well as the time and surface exposed to the sun.

Of particular importance in the increase in cardiovascular mortality due to vitamin D deficiency may be the fact that the role of vitamin D in angiogenesis has also been described. The importance of vitamin D in the development of tumor vasculogenesis, retinal vasculature, or preeclampsia has been demonstrated (57, 78-80). Vitamin D improves the proliferation and differentiation of the myocardial muscle by its action on the genes that control these processes (81-83).

1.6.1. Vitamin D deficiency and the factors of cardiovascular morbidity

1.6.1.1. Obesity

Obesity is clearly an independent factor of cardiovascular events (84, 85). The relationship between vitamin D deficiency and obesity is well known (86, 87). The connection has been described not only in adults but also in adolescents, and the risk of vitamin D deficiency associated with obesity in young adults is even higher than in adults (88). But the association may not be causal. According to a recent review although these two phenomena are connected, weight loss has just a little effect on normalizing vitamin

D level. Nor vitamin D supplementation has any effect on decreasing bodyweight (89). Vitamin D is a fat-soluble protein. It is distributed in the fatty tissues of the body. A bigger proportion of body fat may result in a lower serum vitamin D level. As we can see in obese individuals where lower serum vitamin D levels generally not resulted in disturbances of calcium or bone metabolism (87).

1.6.1.2. Diabetes

Diabetes is also considered an independent risk factor for a higher incidence of cardiovascular events (19). There is growing evidence for an inverse correlation between serum vitamin D levels and diabetes, suggesting that low vitamin D levels may be a major regulator molecule in the development of diabetes. Vitamin D alone is required for proper insulin secretion (17, 20). However, in the development of insulin resistance, β cells attempt to compensate hyperglycemia by increasing insulin production. During this process, the accumulating intracellular Ca^{2+} and free oxygen radicals initiate inflammatory and apoptotic processes in β cells (19). Normal vitamin D levels are also essential to control these inflammatory processes (18, 90). Vitamin D also plays a role in increasing the insulin sensitivity of cells. According to studies by Mitri et al., vitamin D supplementation with insulin increased the glucose uptake capacity of cells in high glucose-treated adipocytes (91).

The cause of the effects of vitamin D in promoting insulin secretion, inhibiting β -cell apoptosis, and increasing the insulin sensitivity of target organs can be found in the influence of genes involved in the processes of glucose and insulin metabolism and production. Many of the genes taking part in the formation of diabetes can be activated by methylation. Vitamin D found to increase the level of demethylase enzymes so it can prevent the hypermethylation of many diabetes-related genes (92-95). It is easy to see from the above that a decrease in vitamin D levels can affect sugar metabolism in a number of ways and contribute to the development of diabetes.

1.6.1.3. Dyslipidemia, atherogenesis

The significance of dyslipidemia is primarily due to its atherogenic effect (96). The role of atherogenesis in cardiovascular disease is evident (97), so is that of dyslipidemia which is linked to obesity. Dyslipidemia is an abnormal level of lipids, cholesterol triglycerides, and lipoproteins forms from the previous two in the serum. These serum fats are classified in the blood according to their density. We can list low density (LDL), very low density (VLDL) ultralow-density (ULDL), and high density (HDL) lipoproteins (98). Many studies already pointed out the association between vitamin D deficiency and dyslipidemia (99, 100). Based on these, low serum vitamin D levels negatively affect the optimal lipid profile. The benefit of vitamin D supplementation on the correction of dyslipidemia is controversial in the literature. The work of Ponda and his colleagues, who reached a similar conclusion in a very large number of cases, can be recalled here (101).

1.6.1.4. Hypertension

Among others one of the most analyzed pathologic connections of vitamin D deficiency is its role in initiation of hypertension (9, 10, 102, 103). Observational studies show clear connections between vitamin D hypovitaminosis and high blood pressure (102, 103). Several different mechanisms may lead to this. Effects of vitamin D deficiency on increasing arterial stiffness (104, 105), acting through the impairment of endothelial function (61, 106), tendency toward atherogenesis (107), and the enhancement of the body oxidative stress (108, 109) all are well-studied processes. Based on the above cited studies lower serum vitamin D levels are associated with elevated augmentation index and pulse wave velocity, and decreased subendocardial viability ratio. Indexes of the arterial function like reactive hyperemia index and endothelium-dependent brachial artery flow-mediated dilation decreased as an effect of low serum vitamin D levels. The explanation of it can be that vitamin D attenuates atherogenic plaque formation by the reduction of the inflammatory processes. It can also affect pathologic wall remodeling through VEGF and tissue matrix metalloproteinases (MMP) (110). Associations between serum vitamin D levels and the activation of renin-angiotensin system (RAAS) can also play an important role in these processes (111, 112). As circulating parathormone (PTH) level is known to be a component of the pathomechanism of hypertension (113), the PTH

and Ca^{2+} levels as a function of serum vitamin D level can also be an explanation. Low serum vitamin D level increases the release of PTH in addition to maintain normal serum calcium level (114). Studies suggest that elevated circulating PTH is associated with high blood pressure (113). Well known phenomena that calcium supplementation reduce the risk of hypertension and cardiovascular disease (CVD) (115). Although it is still not clear whether hypocalcemia alone, or serum PTH and vitamin D levels as well as their effects on the elevation of intracellular calcium are in the background of blood pressure rise. Based on these studies we can conclude that there will be an increased contractility in the presence of catecholamines as well as an elevated basal tone of the arteries in the case of low serum vitamin D level.

1.7. Vitamin D deficiency and major cardiovascular events

Cardiovascular research often discusses the so-called major adverse cardiovascular events (MACE) because they both require special attention due to their severity and they are a well-defined endpoint studying an effect of a condition or physiological factor. The definition of MACE is not uniform in the literature, but in the clinical practice there are usually two forms. These are stroke and myocardial ischemia. I would like to address these two issues separately, as vitamin D deficiency can ultimately lead to these serious diseases through its effects on the predisposing factors described above.

1.7.1. Vitamin D deficiency and stroke

Stroke occupies a prominent place in mortality statistics. The number of people affected worldwide is estimated at 15 million a year (116). Its risk factors include age, race, smoking, as well as sex, dyslipidemia, hypertension, obesity, and diabetes, which are also relevant to the present dissertation (116). It is not clear from the literature that vitamin D deficiency is an independent risk factor for stroke, but at the same time it may lead to it through its role in stroke-leading conditions. If the connection of vitamin D status with the development of stroke is in doubt, a relationship can be easily fixed between vitamin D deficiency and the severity of the stroke developed (117-119). And serum vitamin D levels are considered a predictor of recovery in stroke patients (120). The normal

functioning of cerebral autoregulation can play a huge role both in preventing the development of a stroke and in achieving the best possible long-term outcome after a stroke (121). The quality of cerebral autoregulation is largely determined by the function, contractile and relaxation properties, and histological structure of the carotid arteries. These properties will ultimately determine carotid stiffness, which is an independent risk factor for stroke (122). Based on literature data, a diet that reduces or increases serum vitamin D levels significantly reduces the diameter of the carotid arteries and increases the wall thickness (123). In elderly patients, there is a strong inverse correlation between vitamin D levels and vessel wall thickness in the carotid arteries and the intima-media ratio of the vessel wall (124). Altered relaxation and contractile functions of arterial section of coronary circulation (125, 126), cerebral arteries (41, 127), renal arteries (128), and the aorta (129) by vitamin D deficiency were also confirmed by our research group. According to these studies coronary arteries showed decreased contractility as an effect of thromboxane agonists and decreased relaxation as an effect of testosterone and estrogen. On the contrary, vitamin D supplementation improved the myogenic tone and the ability of relaxation. Vitamin D deficiency enhanced the vascular wall thickness and smooth muscle tone of the anterior cerebral artery (ACA) what can be explained by the decreased expression of eNOS. As a conclusion, vitamin D deficiency (VDD) resulted in an inward hypertrophic remodeling of the cerebral arteries which can lead to a dysfunction of the normal cerebral blood flow. In the renal artery, vitamin D deficiency caused increased phenylephrine-induced contraction. Acetylcholine-induced relaxation was decreased and increased elastic fiber density found. In the same work eNOS staining was decreased in VDD deficient females. In vitamin D deficient circumstances decreased acetylcholine induced relaxation and lower elastic fiber density of the aorta was found (128).

Because of the above facts, our research team also became interested in examining the relationship between carotid artery function and vitamin D deficiency in the adolescent period.

1.7.2. Vitamin D deficiency and myocardial ischemia

Disease of the coronary arteries (CAD) and the factors influencing its development are the most studied phenomena in Western medicine (130). It ranks first in mortality statistics worldwide (131). As with stroke, risk factors for CAD include dyslipidemia, obesity, hypertension, diabetes, age, and sex. Not surprisingly, the study of its risk factors, including its relationship to vitamin D, has long been in the focus of interest (130, 132, 133). Among the most studied cardiovascular effects of vitamin D deficiency is its important role in the ischemic diseases of the myocardium. We have known for 30 years that the receptor for vitamin D can be found in the heart muscle (134). The effects of vitamin D deficiency on the heart and the functioning of the coronary network have since been studied. Studies show a clear association of vitamin D deficiency and the incidence of CAD. In addition to the processes mentioned in the case of stroke, vitamin D deficiency increases the contractile function of the heart muscle and promotes its fibrous transformation (135-137). Several mechanisms may contribute to the formation of CAD in vitamin D deficiency. Vitamin D plays a role in platelet function. The inhibitory effect of platelet aggregation should be emphasized. Vitamin D deficiency has been shown to lead to increased platelet aggregation, a clear risk factor for CAD (138). The influence of endothelial function by vitamin D is also a well-known process. Adequate levels of vitamin D are essential for the adequate synthesis of endothelial nitric oxide (NO), which is essential for proper endothelial relaxation and regeneration (57, 106). Inflammatory processes, atherogenesis, arterial stiffness is also influenced by vitamin D (71).

Until now, the focus of vitamin D-related studies on the coronary system has been on the histological and contractile features of the coronary arteries, their atherogenicity, and their response to inflammation. Our research team has previously used video microscopy to successfully verify network-level changes in the coronary system due to hypertension, aging, or exercise (139-141). Thus, the question also arose as to how vitamin D deficiency affects the development and remodeling of the coronary system. Does vitamin D deficiency cause network anomalies? This suggestion was confirmed by the study that is part of my dissertation (142). This raises the possibility that suffering from vitamin D deficiency during the development of the coronary network may even affect the occurrence of subsequent CAD. This suggestion will be confirmed by later studies.

1.8. Sex differences in cardiovascular morbidity

It is a general observation that men and women have different levels of involvement in cardiovascular disease. In addition, this exposure changes with age (143, 144). It should be recognized that there are physiological factors unique to women, such as menopause, pregnancy, use of contraceptives, polycystic ovarian syndrome (PCOS), that affect cardiovascular risk (145). In recent decades, the effects of sex hormones on cardiovascular events have been studied by several authors (143, 145-148). There is no doubt that it is primarily the sex chromosomes that are responsible for the gender differences in cardiovascular events. The genes located on them control the function and development of the reproductive organs, through this, the amount of sex hormone production (149, 150). The androgen receptor gene located on chromosome X may be of particular importance. The following considerations can be considered when examining age and non-specific disease progression. Steroid hormone receptors are present in most tissues in the body. Based on the metabolic characteristics of steroid hormone, both male and female sex hormones are present in both sexes. Their absolute amount and relative proportions vary with both age and gender. It is also worth highlighting the gender differences regarding MACE.

1.8.1. Sex differences in stroke

It is worth highlighting the gender differences in MACE. The incidence of stroke is higher in men, at the same time, more women are living with the consequences of stroke, and numerically more women are suffering from stroke. The reason for this is to be found in life expectancy. In general, the incidence of stroke in women increases after menopause compared to men and accumulates at an older age due to the longer life expectancy of women. The stroke in men is usually more severe, with more fatalities (151-153).

1.8.2. Sex differences in CAD

More women worldwide suffer from ischemic episodes. Typically, most of this occurs after menopause. Women develop the disease more severely and with a worse outcome at a later age than their male counterparts. Typically, in this older age, women have more risk factors than their male counterparts. Unfortunately, women are more likely to have asymptomatic and NON-STEMI infarction (154, 155).

1.8.3. Sex related alterations in cardiovascular effect of vitamin D deficiency

International data suggest that vitamin D deficiency and supplementation affect cardiovascular risk differently in men and women. In healthy men vitamin D supplementation reduces cardiovascular (CV) risk, although this effect is not observed in women (156).

Based on to the results of the MONICA study, the risk of stroke, CVD, and general mortality increases in middle-aged adults with vitamin D deficiency in women, but this effect is not observed in men (157).

Based on the results of our research group, sex alterations in vascular function and structure in terms of cerebral and coronary, renal arteries in vitamin D deficiency in rodent model can also be observed (125, 126, 128, 158). According to these studies vitamin D deficiency resulted in increased carotid artery contractile reactivity in male rats. Prostaglandin inhibition reduced contraction in female rats' carotid artery, but increased relaxation in male. In the same vessel the number of elastic fibers decreased by VDD in female rats, but not in males. In ACA in female VDD and testosterone treated rats decreased lumen and increased wall thickness detected. In males VDD itself decreased the lumen and increased the wall thickness. Vitamin D deficiency resulted in increased phenylephrine-induced contraction of renal arteries of female rats. Angiotensin II-induced contraction is more sever in male rats' renal arteries if vitamin D is supplemented then females. The intensity of eNOS immunostaining is decreased in vitamin D deficient females in renal arteries than in males.

1.9. Vitamin D deficiency in experimental human and rodent models

In general, human studies are difficult to implement due to several factors. In the case of human studies, it is extremely difficult to form homogeneous study groups, and our results may be affected by the different biometric characteristics of the individuals. It is unethical to use extremely high doses of drugs in such studies, and the different medical histories and living conditions of individuals can show extraordinary differences. In contrast, in the animal models used in the research, where practically all the essential characteristics of the animals can be selected, their general housing conditions and dietary characteristics can be determined, their sex, age can be adjusted, even known extreme doses of drugs that pose a risk to the animal can be used and we can create homogeneous groups. As a result, we can get a cheaper experiment and results that can be evaluated even with a smaller number of individuals. Nevertheless, this obviously has great limitations for human medicine.

The above statement is also true for the studies of vitamin D deficiency. In human experiments, we rarely get information about, for example, the time and severity of VDD, experimental groups cannot be identical by age and connected medical conditions.

When using animal models, we can accurately determine the sex of the animals, biometric data, the amount and duration of vitamin D intake, exposure to sunlight, and these can be generalized to each individual.

Two types of animal models have spread throughout the literature. In the genetic models, genes that are involved in the synthesis of vitamin D, or genes encoding the VDR as well as those that mediate the cellular effects of vitamin D are knocked out, thus ensuring a state corresponding to vitamin D deficiency (159, 160). The model shows the effects of vitamin D deficiency with great certainty, extremely homogeneous groups can be formed with its help, but it is far from “physiological” vitamin D deficiency and does little to model human studies.

The other type to model vitamin D deficiency is the dietary model, where animals fed with a vitamin D free diet what we also used in our recent experiments as well as in our previous studies (108, 128, 129, 142, 161). There are several different vitamin D deficient foods available from companies offering laboratory animal feeds. By using them for different periods of time or by supplementing them with vitamin D, one can easily achieve the desired vitamin D levels. The clear advantage of this method is its low cost and that

it is more like the human situation, as there are also dietary and possibly environmental causes of vitamin D deficiency in the human population.

Normal, low, and high serum vitamin D levels in rat models differ from those established in human studies. Several studies have addressed to establish normal vitamin D levels in rats. Trechsel et al. determined normal 25(OH)D levels in rats at 40 nmol/l (what contributes to 16 ng/ml) (162). At the recommended daily intake, Mirhosseini found the serum 25(OH)D level 17.2 ng/ml in rats after 4 weeks of fed with normal recommended vitamin D intake, 25(OH)D levels of 43.2 ng/ml were described after fed with high dose cholecalciferol contained chow (5000 E/day vitamin D corresponds in human model). Rats on vitamin D-free diet developed 12 ng/ml of serum 25(OH)D in the same study (123). Wilson et al. reported 37-38 ng/ml of serum 25(OH)D level in supplemented animals (163). As a result of vitamin D deficiency Trechsel considered vitamin D deficiency if the serum 25(OH)D level was less than 25 nmol/l (10 ng/ml) (162). The toxic level of serum 25(OH)D in human is 224 ng/ml by Mirhosseini et al. in rat model (123).

Based on Halloran's study, 25(OH)D levels of two weeks old breastfed animals is 10 ng/ml. 8 ng/ml was found at 25 days, and 9 ng/ml at free weeks after weaning (164). 25(OH)D levels of breast fed 4 weeks old Holtzman rats were undetectable and remained at level following an 9-week long vitamin D free diet, while after 30 international unit (IU) daily supplementation of vitamin D increased the serum 25(OH)D level to 9.5 ng/ml and 14.1 ng/ml after 6 and 9 weeks (137).

We planned our experiments based on the results of above-mentioned studies. In our model 4 weeks old Wistar rats right after weaning from mother milk were randomly selected into groups getting vitamin D free and vitamin D supplemented chow. For investigation of gender differences of the contractile function of the carotid arteries in one part of our studies we created male and female subgroups of the two major groups also.

As a result of our methods, normal levels of vitamin D in the vitamin D supplemented group and severe vitamin D hypovitaminosis in the vitamin D deficient group could be achieved (19.66 ± 0.81 and 3.59 ± 0.21 ng/ml) (41, 142, 165). Our results with the applied animal models corresponded to the vitamin D deficiency and normal vitamin D levels expected. Thus, based on the above literature data and the results of our model, we were able to normalize the relatively moderate vitamin D deficiency of rats recently weaned

from mother milk with vitamin D supplementation, while vitamin D free diet remained the vitamin D levels in that low range.

1.10. In Vitro methods used to investigate anatomy and function of arteries

1.10.1. Myography

One of the best-known methods for laboratory examination of the function of small arteries is pressure myography (166), and the wire myography (167). Using pressure myography, examining isolated small vessel segments, our research group has previously published the results registered with this method several times (38, 126, 168). The pressure myography is suitable for the examination of previously prepared vascular sections up to 200 μm in diameter. With its help we can evaluate not only the function of the blood vessels, but also their morphological characteristics. During the method, we can perform our measurements by cannulating both ends of the branchless straight vessel, perfusing it, and placing the vessel in an organ bath. The intraluminal pressure can be adjusted by varying the perfusion. We can provide constant temperature and physiological condition in the organ bath. The effect of different vasoactive pharmacons can be tested both by injection into an organ bath and by perfusion into a blood vessel. High resolution pictures can be taken with the help of a microscope, which can be analyzed by examining the effects of perfusion pressure and pharmacons, by analyzing the diameter and wall thickness of the vessels. The intraluminal pressure can be adjusted by varying the perfusion.

With the wire myograph which we used in our research isometric contraction of different arteries can be measured. The technique has been used successfully since the late 1970s (169-171). Similar to the pressure myograph method, a ring element of the test vessel section is placed between the pressure measuring pins. The device measures the isometric contractile force of the vessel wall at a specified fiber length. The reference contraction of the vascular section applied to the device is determined with K^+ solution, and we can later compare our results to this registered value. Various pharmacons that increase vasoactive contraction or promote relaxation are placed in the organ bath at different concentrations. The effects will be a change in the tension exerted on the device

by the vessel wall. The measured data is analyzed by computer software and displayed using graphical methods. Previously our research group also used this technique to publish interesting data of measurement of different types of arteries like aorta, renal arteries, or carotid arteries (129, 172).

1.10.2. Histology

In vitro studies include immunohistochemical and histological processing of blood vessels. Using the method, we obtain semiquantitative information about the tissue structure of the vessel wall, the proteins found in the vessel wall. According to the classical method, the tissues to be examined are dehydrated after removal and then fixed in paraffin blocks. With the help of a microtome, we can make thin sections, which are treated with different paints, depending on the type of material we want to see. A special method for examining proteins is immunohistochemistry, in which the test antigen is labelled with an antibody and stained. Our research group previously successfully used the histologic and immunohistochemistry staining to analyze the alteration of the structural and functional changes of the vessel (140, 168).

1.10.3. Video microscopy

To understand the optimal structure of a vascular network, we need to look back to Murray's work (173, 174). Deviation from the ideal structure is clearly not advantageous and reduces the efficiency of the network. However, the formation of the geometric structure of the network is a much less studied element of the research. This is why this technique has come to the forefront of our research (139-142, 175).

Techniques for examining the coronary network have different limitations. Histological examinations are very useful, but they only give a cross-sectional view of the blood vessels, they do not provide information about their complexity or network structure. However, the function of the blood vessels cannot be examined by this method either.

Although myographic examinations provide information on the contractile and relaxation function of blood vessels, they also do not provide a comprehensive picture of the network properties of blood vessels. Although the imaging studies that can be used in

the clinic inform about the complex course of the vessels, the properties of the networks and show real physiological conditions, their resolution is not good enough to judge the more detailed network structures, branches, and wall thicknesses.

However, coronary mapping and video microscopy meets all of these criteria. The method of the video microscopic technique itself and the micro preparation of the coronary artery system required for it have already been developed in rodent model (175). The increasing application of the technique has made it possible to study the effects of various variables on the coronary network, considering all the aspects detailed above. To be able to describe the properties of the coronary network, examining the branch system and its elements, wall thickness, length up to 80 μm of diameter under physiological conditions. Using the technique, our research team has previously showed the effects of high blood pressure, aging, or physical training on the structure of rat coronary networks (139-141). In our study the micro preparation of the left anterior descending artery (LAD) and the mapping of the resistance coronary artery network has been made as previously shown (175). All investigated coronary system remained intact during the process. 80 μm large branches and segments became visible. The orifice was cannulated, the LAD system recorded in physiological circumstances during perfusion. All bifurcations segments and 50 μm long cylindrical unit of the vessels presented in a coordinate system, where X-axis draw has been drawn connecting the points of the orifice and the apex of the heart. Segments length, diameters, branching, the Murray's law, hemodynamically disadvantageous network anomalies, number, position and the thickness of the resistance artery wall of the 50 μm long ring units were measured (142).

2. OBJECTIVES

Cardiovascular-related deaths, hypertension, and myocardial ischemia are associated with vitamin D deficiency. Both coronary arteries and carotid arteries play an important role in the cardiovascular risk. In our studies related to the dissertation we investigated how vitamin D deficiency alone, and vitamin D related physiological processes can modify the geometry, segments and branching of the LAD coronary artery network analyzed by video microscopy technique. Furthermore, how vitamin D deficiency affected the vasoconstrictive response of the carotid artery using wire-myograph method and what sex differences are found in this process.

To examine coronary vessels, male Wistar rats selected into male vitamin D deficient (MVD⁻, n=10) and male vitamin D supplemented (MVD⁺, n=8) groups. To examine carotid vessel, male and female Wistar rats were randomly selected into the following groups: male vitamin D deficient (MVD⁻, n=11-13), male vitamin D supplemented (MVD⁺, n=11-13), female vitamin D deficient (FVD⁻, n=11-13) and female vitamin D supplemented (FVD⁺, n=11-13). Vitamin deficiency achieved by a vitamin D free diet vitamin D free Lab Rat/Mouse Chow, (<5 IU/kg Vitamin D₃, Ssniff Spezialdiäten GmbH, Soest, Germany). Animals of VD⁺ group were fed by a standard laboratory diet (1000 IU/kg of vitamin D) for 8 weeks. And additional 500 IU cholecalciferol on week 2, and weeks 4, 5, 6, 7 and 8, weekly dose of 140 IU/100 g administered through a gavage cannula (128). After 8 weeks, LAD network was analyzed by video microscopy technique and carotid artery was analyzed by wire-myograph technique. Histology was made to analyze the effect of vitamin D on the detailed structure of the wall of the carotid artery.

In this dissertation I sought the answer for the following questions:

- 1) Are dietary alterations of vitamin D can change the geometry, branching, and distribution of arteries of different diameters of the LAD network in rodent model?
- 2) Are there any sex-specific differences in the contractility of the carotid artery associated with different serum vitamin D levels?
- 3) If there are, are these effects comes with related sex-specific alterations of the histological structure of the carotid arteries of the rat?

3. RESULTS

3.1. Coronary artery mapping

3.1.1. Biometric data. Body and heart weight, arterial blood pressure

Vitamin D deficiency and supplementation for 8 weeks long therapy did not affect the biometric data. Differences of body weight (MVD⁻: 481±15 vs. MVD⁺: 477±19 g), heart weight (MVD⁻: 1.37±0.04 vs. MVD⁺: 1.32±0.08 g), was not appeared among the groups. Blood pressure was observed invasively by measuring it in the right carotid artery and it did not show any significant alteration between the two groups (mean arterial pressure: MVD⁻: 95.39±4.35 vs. MVD⁺: 88.18±6.57 mmHg, in Nembutal anesthesia).

3.1.2. Segments analysis

Observing the subsurface network, the segments and branching of coronary resistance artery up to an outer diameter of 80 micrometers showed no difference between the study groups (MVD⁻: 210 and MVD⁺: 224 segments, normalized in 8 animals). A major difference was the significantly larger lumen surface area of the primary branches in the MVD⁺ group. Lumen diameters did not differ during the next branching steps (**Fig. 1A**). Segments of order 1 and 4 were significantly, 2 and 3 were slightly longer in MVD⁻ rats (paired t-test (**Fig. 1B**)). Interestingly, branches of 11-12 order were showed up only in the MVD⁺ group (**Fig. 1A and 1B**).

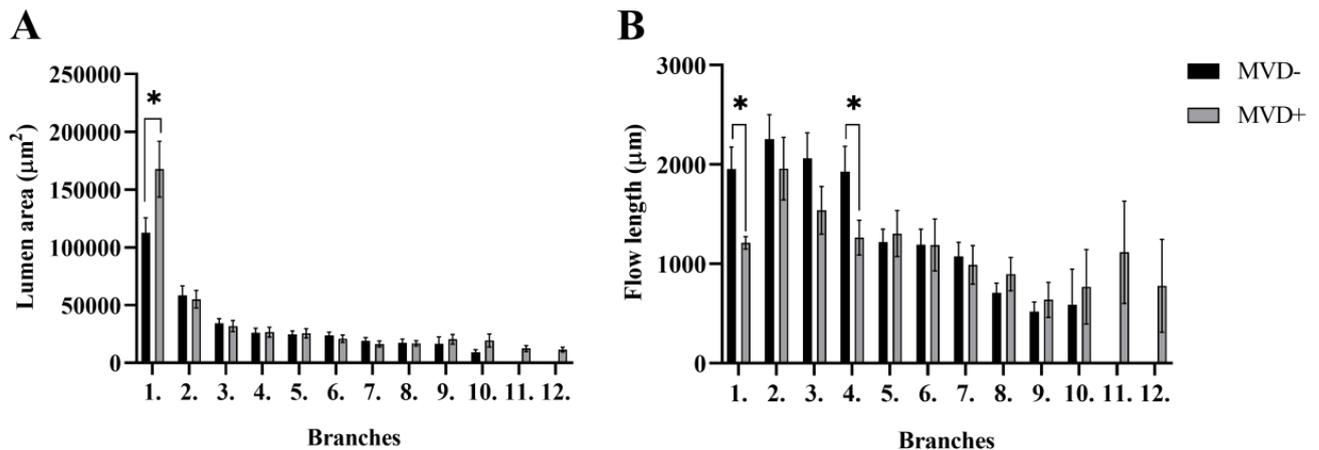


Figure 1. Segments analysis

(A) cross-sectional area of the segments lumen from the MVD⁻ and MVD⁺ animals. The cross-sectional lumen area of the first order branches was significantly bigger in MVD⁺ group. (B) The lengths of the segments of MVD⁻ and MVD⁺ animals. The 1. and 4. order segments are lengthier in VD⁻ rats. Values are means \pm SEM. n=10 in MVD⁻ and n=8 in MVD⁺. Two-tailed unpaired Student's t-test and Mann-Whitney-test, *P<0.05 MVD⁻ vs. MVD⁺ (142).

3.1.3. Analyzes of the branches

One of the guiding principles of microvascular geometry is that smaller branches deviate more from the axis of the main branch than larger secondary branches. So, the branch angle between the branches increases with increasing asymmetry in the diameters of the daughter branches. Analyzing the vitamin D substituted networks, this is clearly visible (**Fig. 2A**, significant by Pearson correlation). This association is lacking in vitamin D deficient animals (**Fig. 2A**).

Another feature of microvascular bifurcation geometry is that the lumen diameter of the daughter branches conforms to Murray's law. **Figure 2B** shows that the bifurcation of the study groups was met the criterion exactly of Murray's law.

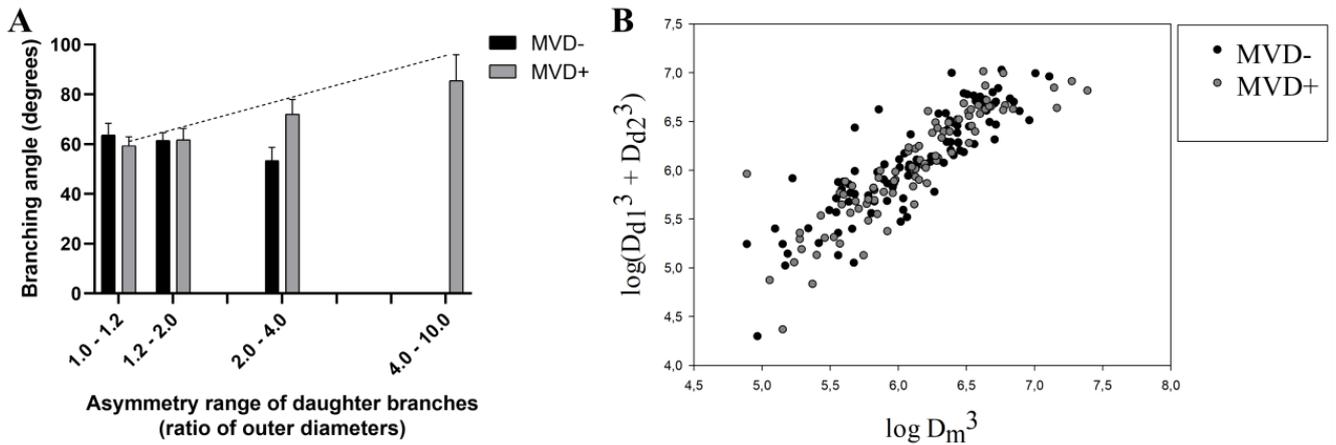


Figure 2. Analysis of branching

(A) Range of asymmetry of the secondary branches (ratio of outer diameters). The index (with increasing branching angle) significantly elevated in MVD+ groups only ($p < 0.05$ Pearson correlation, Values are means \pm SEM). $n=10$ in MVD- and $n=8$ in MVD+). (B) The Murray's law. Branching are obeying the Murray's law (142).

3.1.4. Abnormalities

Vitamin D deficiency did not change the amount of morphological deviation such as parallel running, track breakage, multiple segments branching, or vessel tortuosity (**Table 1**).

Table 1. Number of morphological abnormalities of the coronary networks

Morphologic deformity	MVD-	MVD+	Chi ² (X ²) probe significance level
Parallel running	2	3	0.65
Broken course	7	6	0.74
Multiple branching	11	8	0.49
Tortuosity > 8	7	5	0.56
Sum of all deformities	27	22	0.48

Overall number (normalized for 8-8 rats) of morphological deformities demonstrated no differences in the coronary resistance artery network of the left anterior descending coronary artery of MVD- and MVD+ groups (142).

3.1.5. 50 μm long cylindrical ring unit analysis

MVD+ animals had a slightly richer coronary resistance arterial network compared with MVD- rats (**Fig. 3 and 4**). Dividing the entire network into ring units of 50 μm , there were significantly fewer such units in MVD- group. (6365 vs. 6602, overall data, normalized to 8 study animals with a $p < 0.0374$ χ^2 probe). **Fig. 4A** shows that in MVD+ animals, increased number of vascular units was present in the outer diameter range of 100–300 μm . The only exception is the 250 μm diameter ring units. The peak of the histogram in MVD- animals shifted a little towards 250 μm from 200 μm observed in substituted animals. MVD- animals had significantly bigger amount of larger diameter ring units in the range of 400–550 μm . At the same time at this range of diameter the ring units showed wall thickening. Thickness of the vessel wall thickening was bigger in MVD+ animals in the most common diameter range of 200–300 μm (**Fig. 4B**).

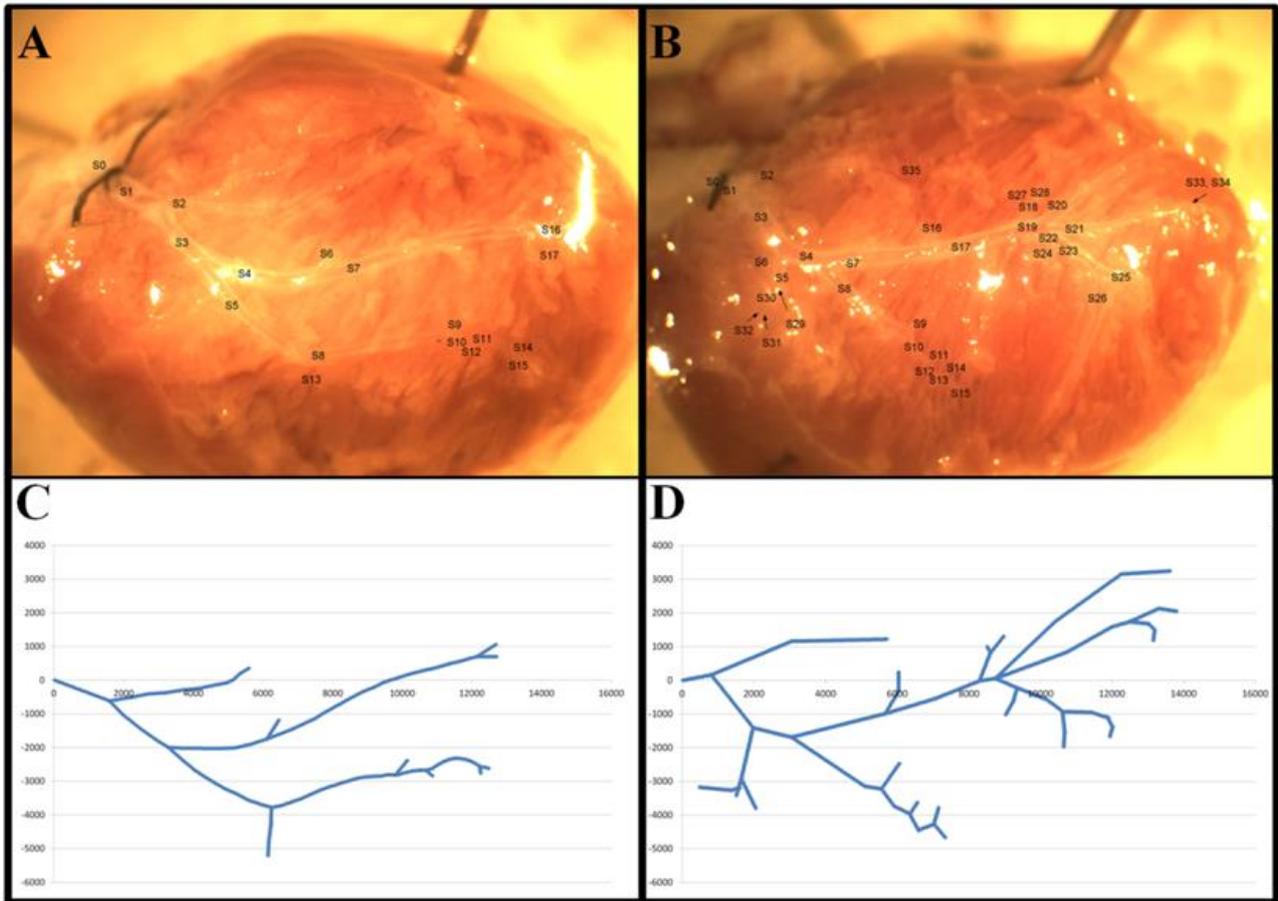


Figure 3. (A-B) Representative images made by video microscopy. Segments and branches are numbered. (C-D) A representation of the entire system in coordinate system is shown.

Harsh difference between the two experimental study groups in the network density of the LAD coronary artery, shown in the image and the associated coordinate system of a typical vitamin D deficient rat (A,C) and a typical vitamin D supplemented rat (B,D) (142).

The next interesting idea was to determine the exact geometric distribution of this numerical deviation. The heat-map histogram in **Fig. 5** shows that a new population of 250 μm diameter ring units appears at a flow distance of 6-9 mm from the orifice in vitamin D deficient animals, while a decrease of 350 μm is observed at the same sites. In vitamin D deficient rats, units of 150-200 and 300 μm are almost absent at a flow distance of 10–15 mm.

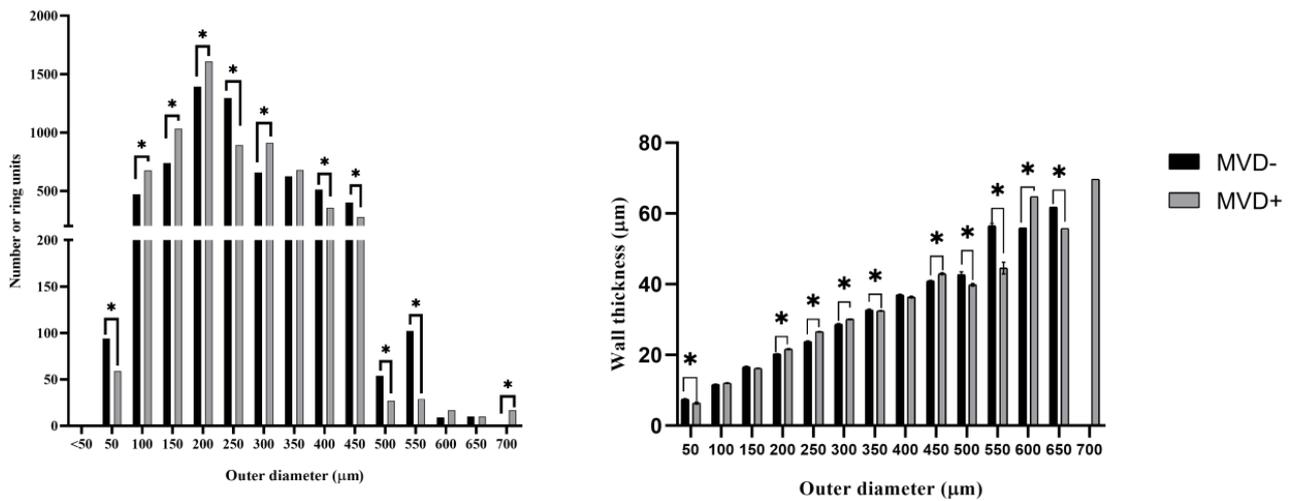


Figure 4. Analysis of ring units

(A) Number of 50 μm ring units as a function of different diameter ranges. In MVD- networks, the number of rings decreased in the range of 100-300 μm (except at 250 μm), and the number of rings increased in the range of 400-550 μm. Normalized for 8-8 rats. Significantly different with the Chi2(X^2) probe ($p < 0.05$). (B) Wall thickness of 50 μm ring units as a function of different diameter ranges. Wall thickness was increased in the 50, 350, 500, 550 and 650 μm range in MVD- group. However, the wall thickness was bigger in the 200, 250, 300, 450 and 600 μm range in MVD+ group. Values are means \pm SEM. $n=10$ in MVD- and $n=8$ in MVD+. Mann-Whitney-test. * $P < 0.05$ MVD- vs. MVD+ (142).

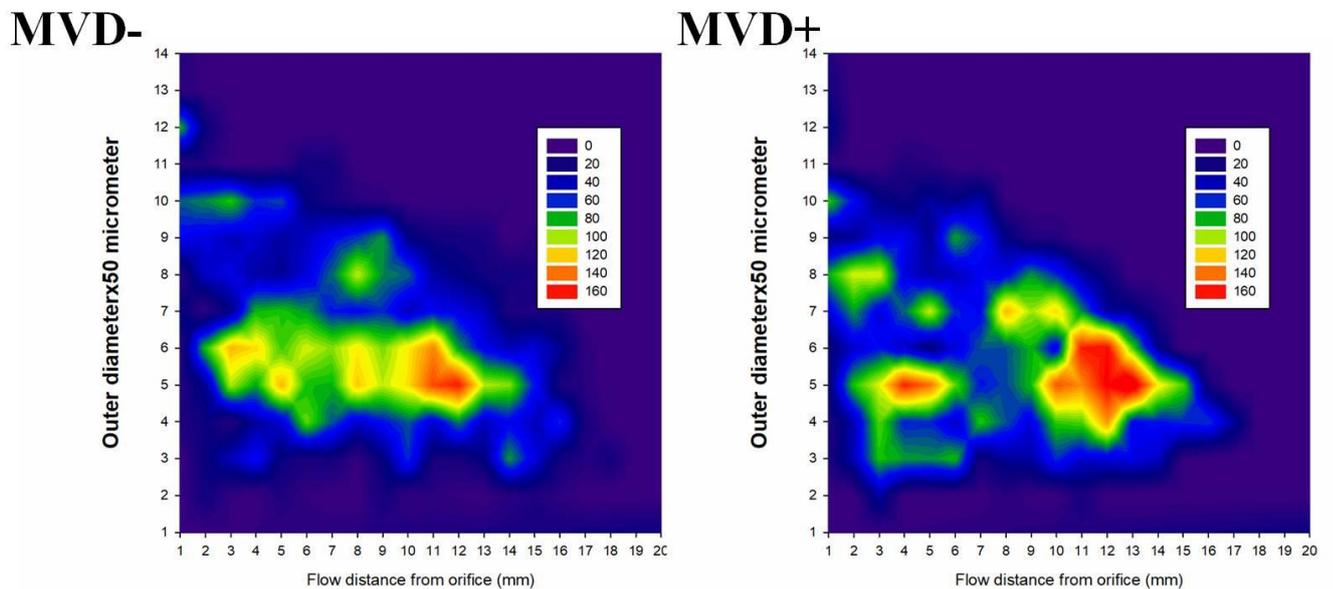


Figure 5. Frequency of ring unit (color coded) for different diameters and flow distances from the orifice

In MVD- rats a new population of 250 μm units appear in a 6-9 mm flow distance from the orifice, while at the same locations there is a diminishment of 350 μm units. In MVD+ rats, 150-200 and 300 μm units are almost missing at 10-15 mm flow distances (142).

3.2.1. Phenylephrine-induced contraction of carotid arteries

Sex alterations in phenylephrine-induced contraction can be seen at phenylephrine concentrations of 10^{-6} mol/L, male rats showed stronger contraction, regardless of the status of serum vitamin D level. In general, vitamin D deficiency resulted in more pronounced contractions induced by phenylephrine (**Fig. 6A**). At phenylephrine concentrations of 10^{-7} mol/l, carotid arteries of male, vitamin D deficient rats showed an enhanced contraction compared to female animals. In addition, female arteries from the vitamin D supplemented study group showed decreased contraction. At a phenylephrine concentration of 10^{-6} mol/l, the carotid arteries of male rats from the supplemented group showed decreased contraction than vessels of male rats with vitamin D deficiency (**Fig. 6B**).

To elucidate the role of eNOS and cyclooxygenases, in the characteristics pointed out above in phenylephrine-induced contractions, the study was repeated in the presence of L-NAME and indomethacin and their combination. L-NAME forced the contraction in all study subgroups. However, indomethacin did not alter the reactivity in the presence of L-NAME in most groups, except for vitamin D deficient males, where it further enhanced the contraction compared with the effect of L-NAME alone. Indomethacin reduced the force of contraction only in female vitamin D supplemented rats, which suggests that vasoconstrictor prostanoids play a significant role in these animals (**Fig. 7A – D**). The lack of effect of indomethacin in male, vitamin D supplemented and female vitamin D deficient study groups suggest sex- and vitamin D dependent regulation of prostanoid induced vasoconstriction.

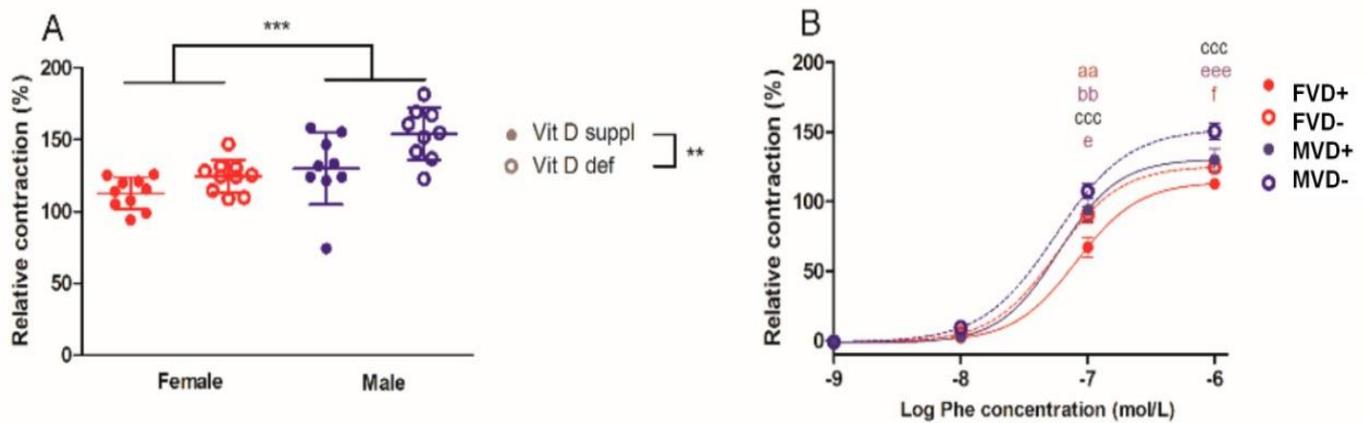


Figure 6. Contraction ability of isolated carotid artery segments

(A) phenylephrine (Phe)-induced contraction in the four experimental groups at 10^{-6} mol/L Phe concentration. Male sex and vitamin D deficiency were associated with more pronounced relative contraction. Data are shown as individual data points; horizontal lines represent mean \pm SEM. Two-way ANOVA; factors: sex, vitamin D status. **: $p < 0.01$, ***: $p < 0.001$. (B) Phe-induced contraction. Male rats showed significantly increased contraction compared to females at Phe concentration of 10^{-7} mol/L independently from vitamin D status. Vitamin D deficient female vessels had stronger contraction compared to their vitamin D supplemented counterparts. At Phe concentration of 10^{-6} mol/L, MVD⁻ arteries contracted more than FVD⁻ ones. Vitamin D deficient male rats showed increased contraction compared to their vitamin D supplemented counterparts. Data are shown as mean \pm SEM; $n = 9-11$ in each group; repeated measures ANOVA, Bonferroni's post hoc test; aa: $p < 0.01$ FD⁺ vs. FD⁻, bb: $p < 0.01$ FVD⁺ vs. MVD⁺, ccc: $p < 0.001$ FVD⁺ vs. MVD⁻, e: $p < 0.05$ FVD⁻ vs. MVD⁻, eee: $p < 0.001$ FVD⁻ vs. MVD⁻, f: $p < 0.05$ MVD⁺ vs. MVD⁻ (161).

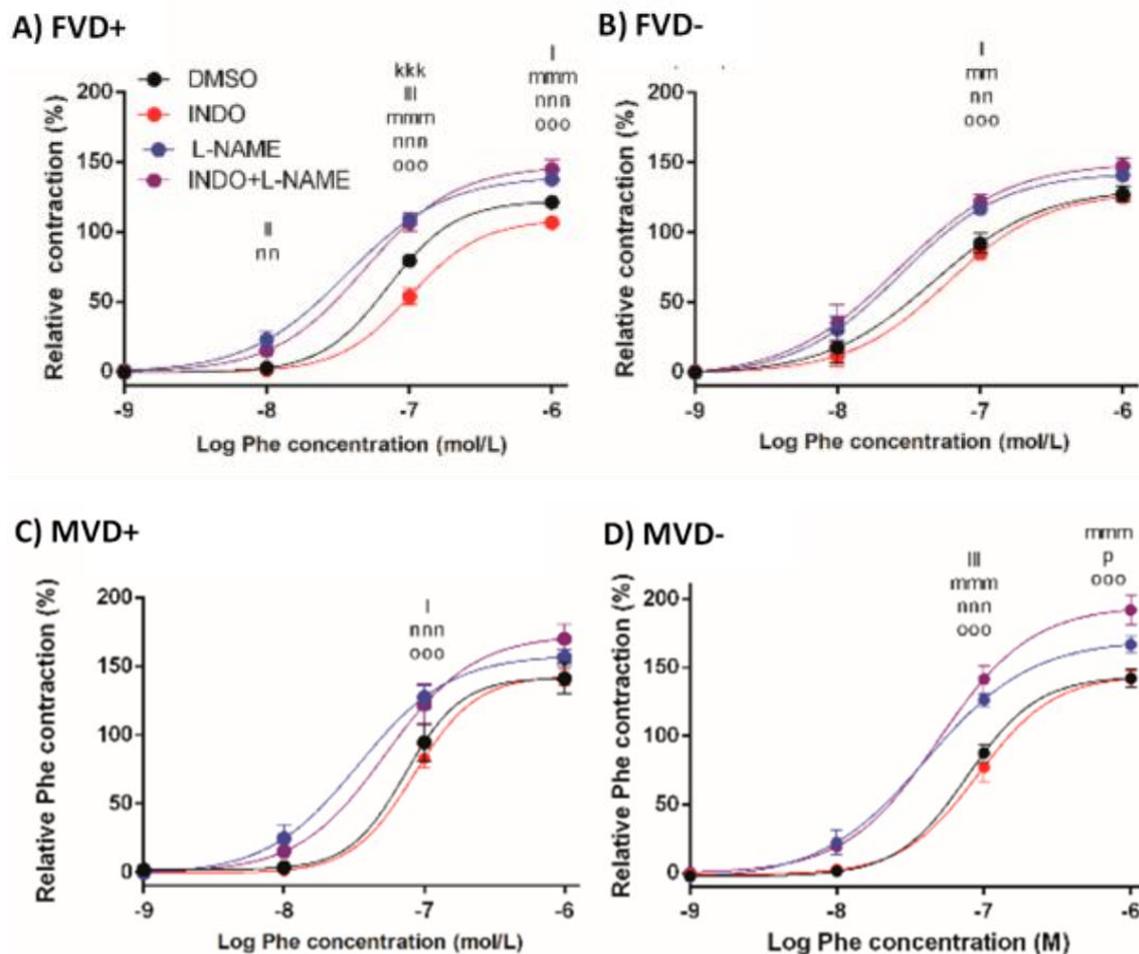


Figure 7. Contraction ability of isolated carotid artery segments in the presence of inhibitors Phe-induced contractions in the presence of L-NAME and/or indomethacin (INDO) or their vehicle DMSO (A) in female vitamin D supplemented rats (FVD+) (B) in female vitamin D deficient rats (FVD-), (C) in male vitamin D supplemented rats (MVD+) and (D) in male vitamin D deficient rats (MVD-). L-NAME increased the level of contraction in all experimental groups. Co-incubation with INDO further augmented the contraction only in MVD- rats. INDO itself decreased the degree of vascular reaction only in the FVD+ group. Data are shown as mean \pm SEM; n = 5–11 in each group; repeated measures ANOVA, Bonferroni's post hoc test; kkk: $p < 0.001$ DMSO vs. INDO, l: $p < 0.05$ DMSO vs. L-NAME, ll: $p < 0.001$ DMSO vs. L-NAME, mm: $p < 0.01$ DMSO vs. INDO+L-NAME, mmm: $p < 0.001$ DMSO vs. INDO+L-NAME, nn: $p < 0.01$ INDO vs. L-NAME, nnn: $p < 0.001$ INDO vs. L-NAME, ooo: $p < 0.001$ INDO vs. INDO+L-NAME, p: $p < 0.05$ L-NAME vs. INDO+L-NAME (161).

3.2.2. Vitamin D status and gender induced histological alterations of the carotid arteries

Vitamin D deficiency decreased the number of elastic fibers in isolated carotid arteries of female animals significantly but not in males. This can be a result of the marked, but

not significant decrease of elastic fiber staining of the male carotid arteries (**Fig. 8A – 8B**). On the other hand, vitamin D deficiency decreased the staining intensity observed after smooth muscle actin (SMA) immunolabeling of carotid arteries of male animals. This change cannot be seen in females (**Fig. 8C – 8D**). Thromboxane A2 receptor (TP) showed a marked increase of immunopositivity induced by vitamin D deficiency in male animals but not in females (**Fig. 8E– 8F**). Nitrate stress characterized by tyrosine nitration was less strong in both male groups compared to female vitamin D deficient animals (**Fig. 8G – 8H**).

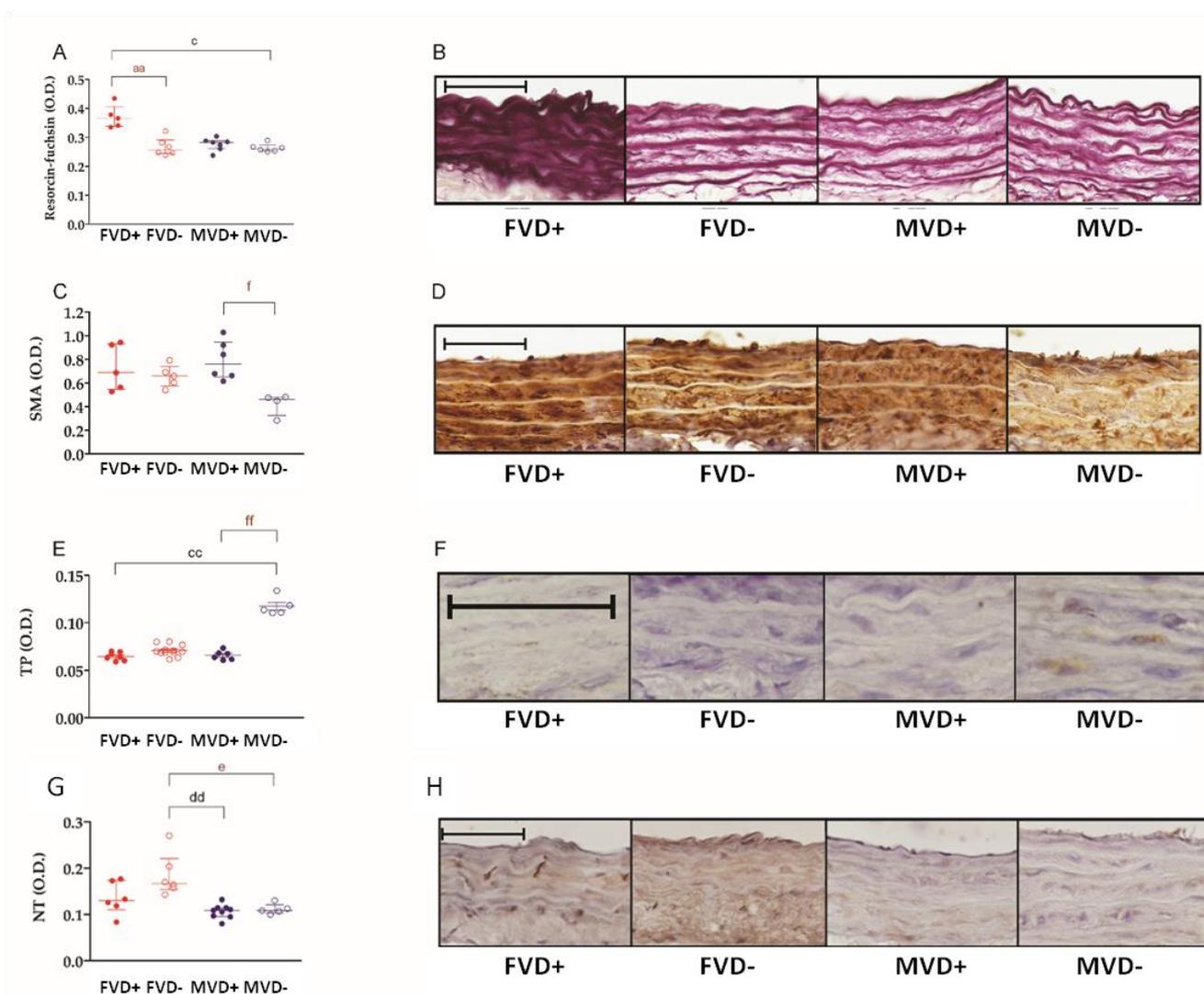


Figure 8. Histological changes of the carotid arteries

(A) Elastic fiber density of carotid artery segments. Tissue sections were stained by the purple-colored resorcin-fuchsin stain. Vitamin D deficient female and male arteries showed significantly lower optical density than vitamin D supplemented female vessels. (B) Representative images of resorcin-fuchsin-stained carotid artery sections. (C) Alpha smooth muscle actin (SMA) immunohistochemical labelling intensity in the media layer of carotid arteries. In vitamin D deficient male rats, the measured optical density was significantly lower compared to vitamin D supplemented male animals. (D) Representative images of carotid arteries stained against SMA. (E) Thromboxane A2 receptor (TP) density of carotid arteries. Vitamin D deficient male rats showed higher receptor density compared to vitamin supplemented male and female animals. (F) Representative images of TP-stained vessels (G) 3-Nitrotyrosine (NT) staining intensity of carotid arteries. Both male groups showed lower positivity than vitamin D supplemented female rats. (H) Representative images of NT-stained carotid artery sections. Specific immunohistochemical labelling was visualized by the brown-colored diaminobenzidine, while blue-colored hematoxylin served as counterstaining. Scale bars show 50 μ m. Data are presented as individual data points and lines represent the median (IQR); Kruskal–Wallis test with Dunn’s post hoc test; aa: $p < 0.01$ FVD+ vs. FVD-, c: $p < 0.05$ FVD+ vs. MVD-, cc: $p < 0.01$ FVD+ vs. MVD-, dd: $p < 0.01$ FVD- vs. MVD+, e: $p < 0.05$ FVD- vs. MVD-, f: $p < 0.05$ MVD+ vs. MVD-, ff: $p < 0.01$ MVD+ vs. MVD- (161).

4. DISCUSSION

Research on the cardiovascular effects of vitamin D has come to the fore in recent decades. The close association of vitamin D deficiency and adverse cardiovascular events has become clear. However, in many comparative studies, vitamin D administration does not clearly compensate this effect (9, 66, 67, 71, 72, 74-76, 176). This highlights that the effects of vitamin D that influences cardiovascular mortality and morbidity have not yet been fully understood.

Vitamin D deficiency affects a number of factors involved in the development of known cardiovascular diseases (177). It is proved to be associated with hypertension (178, 179), obesity (87, 180-182), insulin resistance, (183, 184), or dyslipidemia among others (185).

One of the hypotheses of the present dissertation was that changes in serum vitamin D levels play a role in shaping, structure, and geometry of the LAD network. Our other hypothesis is that, as described in other vessels, vitamin D also affects the contractile functions of carotid arteries, which play a key role in cerebral blood supply.

Revealing the histological changes caused by vitamin D deficiency and supplementation in different tissues have also been a targeted task of our research group (125, 126), part of the results are included in the present dissertation.

4.1. Coronary artery mapping

In our work analyzing coronary networks, we paid special attention to the recording of biometric data, the examination of blood pressure, body, and heart weight.

The relationship between obesity and vitamin D is a known entity. However, the relationship is far from being causal. Due to the fat solubility of vitamin D, it is able to be distributed in a larger amount of body fat in the case of obesity, so that the decrease in serum levels in obese individuals can be only a relative and not an absolute decrease in vitamin D levels. This is supported by the observation that a decrease in vitamin D levels in obesity is not necessarily associated with problems of the bone metabolism (87). In our studies, we did not find any differences in the body weight or heart weight of the animals regarding vitamin D support or deficiency. Thus, the vitamin D levels provided by the model can be considered as absolute values. Vitamin D is also known to affect

myoproliferative processes, affecting gene expression in myocytes and the renin-angiotensin system (81-83). At the same time, we did not find any difference in heart weight in our study. However, these similarities simplified the comparability of coronary networks of the study groups.

For the technical implementation of our research examining network anomalies and complex geometry of LAD, we used micro preparation of LAD and video microscopic analysis (175). Our research team has also used the technique in a number of previous studies, which can be considered an accepted method of examining the geometry of the coronary resistance artery system (139-141). Using the technique, we can get a complete picture of the course and branching of the coronary network, and we can examine the diameter and wall thickness of the segments under physiological conditions by observing the myogenic tone of the vessels. The method provides an accurate and detailed mapping of the coronary network properties seen during physiological circumstances.

The geometrical properties of the formed coronary network ensure an adequate and sufficient supply of oxygen for all parts of the heart muscle tissue to meet their unique needs. Many factors play a role in the development processes of the coronary network (186). The structure of the network must be hemodynamically advantageous and must have a sufficient density to deliver nutrients to the cells. At the same time, the flow resistance must match the needs. The number, length, diameter, wall thickness, and distribution of the segments that make up the network, the properties of the bifurcations, the distance of the network elements from the origin of the vessels, and the curvature of the cavity all contribute to the proper functioning of the system. Based on the literature data of the role of vitamin D in vascular remodeling (83, 186), previously we expected that we find differences in the structure and properties of coronary networks between the malnourished and vitamin D supplemented groups. These data reinforce our notion on the role of vitamin D in arteriogenesis. Namely, the vitamin D deficient state networks will be hemodynamically more disadvantageous than the networks of animals receiving vitamin D supplementation. We could expect to find differences in the structure and properties of coronary networks between the vitamin D free and vitamin D supplemented groups, given the role of vitamin D in arteriogenesis. It was further hypothesized that the video microscopic technique would be suitable to detect these differences. The universal characteristics and geometrical properties of the coronary network have been studied in

several models (187-190). In the previous work of Murray et al. and Zamir et al. demonstrated the key factors of hemodynamically proper vascular systems (173, 174, 191). Using the experience of these works, we considered branches of multiple segments, the parallelly running vessels, the broken course, and the bigger tortuosity as hemodynamically unfavorable deviations. All such morphological aberrations can be expected resulting in a sub-optimal hemodynamic situation. In our previous studies we demonstrated the role of hypertension in the development of these anomalies on rat coronary vasculature. The role of high pressure in the appearance of network anomalies was clearly demonstrated in these studies (139). Our recent studies have shown that Murray's bifurcation laws hold true despite vitamin D status (142), as we previously found for hypertension, physical activity, and aging by analyzing such networks (140, 141).

In our study examining the relationship between vitamin D levels and coronary network properties, we did not find differences between the study groups in number of coronary anomalies, as expected from our studies of the effects of aging and hypertension (139). Blood pressures did not differ between the study groups. All this may suggest that despite that vitamin D has been shown to affect the vasculogenesis (78, 79), vitamin D deficiency alone cannot form unfavorable network structures. However, hypertension, which is associated with several physiological processes (137, 178, 192) connected with vitamin D deficiency, may accelerate development of these pathological abnormalities in the long run. A previous study by Weishaar et al. examined the association between calcium and creatine phosphokinase and hypertension in relation to vitamin D deficiency in a rodent model. Vitamin D deficiency significantly increased arterial blood pressure from week 2 to week 6 of the vitamin D deficient diet but did not cause a blood pressure difference between weeks 7 and 9. The present study of our research group also showed corresponding blood pressure values. Based on the work of Weishaar (137), the authors identified disturbed Ca homeostasis due to vitamin D deficiency as a possible cause of the increase in blood pressure between 2 and 6 weeks. However, hypertension associated with long-term vitamin D deficiency and its causes have an extensive literature (9, 66, 176, 178, 179).

During our studies, we examined the branches of the coronary networks with special attention. We were able to conclude that the increase in bifurcation asymmetry in vitamin D deficient animals was not accompanied by an increase in branching angles, as observed

in the vitamin D supplemented group. This difference can be considered hemodynamically disadvantageous. However, the lack of vitamin D did not elicit deviation from Murray's law, and the ratio of the inner diameters of the mother branches and daughter branches was not altered by different shear forces due to branching angles, and they met physiological expectations in both groups. After micro preparation of the networks up to 80 μm in diameter, the number of segments statistically was the same in the two groups. However, there were several differences in the network geometry of the two groups. Analyzing the major segments after the first bifurcations, we found significantly higher lumen diameters in the MVD+ group, allowing a greater blood flow at this level. However, for the 1-4 order branches, we found statistically longer segments in the MVD- group with increased wall thickness and reduced lumen cross-section, which may result in more vascular resistance at this stage of the networks. It is an interesting observation, and it is important to point out that during preparation up to 80 μm in MVD+ animals 11.-12. order branches could also be observed but this cannot be seen in MVD- animals. All this confirms our inference, following a primary review of native networks, that MVD+ animal networks are denser. According to Poiseuille's laws the length of a vessel section is in direct proportion to its lumen area, and the number of connected elements in the system in parallel, the number of branches, is inversely proportional to the total hemodynamic resistance of the system.

Interpreting the differences described above, it is suspected that the coronary network resistance of animals in the MVD+ group may be lower than the vascular resistance of animals in the MVD- group.

In order to provide optimal oxygen and nutrient supply to the tissues, the body regulates the specific organ-specific tissue perfusion through various mechanisms. Prominent examples of this are cerebral autoregulation and physiological reactions of cerebral vessels to hypertension (193, 194), in which the circulatory system is able to adapt to altered conditions by regulating myogenic tone to provide the necessary perfusion. Numerous examples show this, such as the response to physical activity (195), or vascular responses to chronic hypertension in the retina of the eye (196). Vascular histological abnormalities associated with diabetes are also well known (197, 198). Taking all this into account and knowing the literature data, the network differences found to increase resistance in vitamin D deficient animals in our experimental model, even if no significant

difference in hypertension was found after 8 weeks in agreement with Weishaar's work (137), can also be to the initial effects associated with emerging hypertension.

Following the above analyzes, our study was continued with a more detailed analysis of the network. We divided our LAD system into 50 μm long ring unit elements, examining the properties of these elements, their spatial location, and determining their number. As mentioned above, heart weight did not differ between the two groups. However, the number of vitamin D supplemented animal networks showed a significant increase in the number of ring elements, which led to a higher ring element density in the networks. The lower number of ring elements in vitamin D deficient animals is mainly due to the significantly smaller number of ring elements with a diameter of 150-200 μm . It should be noted that these networks were found to be richer in network elements with a diameter of 400–550 μm , but this numerical difference did not compensate the decrease in the number of ring elements for the entire network.

An increase in the extra distance for each ring element was observed in the MVD+ group (Extra distance = Flow distance - real distance from the orifice). Previous studies on some special vascular networks, such as retinal vessels, have clearly evaluated the increase in extra distance as a sign of hypertension (196, 199). Taking into consideration that no other parameters in our study indicated that the networks showed differences suggestive of hypertension in the MVD+ group, this difference is considered to be a consequence of the richer network.

The distribution of the ring units of the network of different diameters as a function of distance from the rising of the LAD also showed differences between the two groups. The number of medium and small diameter ring elements in the regions farther from the orifice showed a clear increase in the MVD+ group. This may also mean better tissue perfusion. The wall thickness of each ring element was found to be increased in the MVD+ group in the 100-300 μm diameter ring elements, while this difference was observed in the MVD– group in the 400-550 μm diameter and less than 50 μm diameter groups compared to the values measured in the other therapeutic group.

The histogram of the diameter distribution of the 50 μm long ring elements of the networks shows characteristics. In the MVD+ group, the highest density on this histogram is found for ring elements with a diameter of 200–250 μm . We may interpret this as a hemodynamically helpful property of the network.

In previous studies of our research group (108, 126), in which coronaries with a diameter of 200 μm were studied with pressure myography, we found decreased spontaneous muscle tone in vitamin D deficient states, and the walls of these vessels were thicker and the lumen was narrower in complete relaxation. In our present study, we were able to examine the entire coronary network under perfusion pressure. In the coronary network of vitamin D substituted animals, we observed a greater wall thickness than arterioles with a diameter of 200–300 μm , which may be due to the higher myogenic tone in our model of better modeling of physiological conditions in response to physiological intraluminal pressure.

4.2. Carotid arteries

Another focus of our research was the study of carotid arteries, which play a prominent role in brain autoregulation. We were interested in how the contractile functions of these vessels develop in vitamin D deficient and supplemented animals, whether there is a sex difference, and whether the differences seen are followed by specific histological alterations among the study groups.

According to our studies of rat carotid arteries, based on the evidence of our myographic measurements, the carotid arteries showed sex differences in contractility under vitamin D deficient and supplemented conditions. Histological examinations of the vessels also showed specific differences in the studied groups (161).

Based on literature data, it seems clear that arterial function is affected by sex. This observation has already been described for carotids in mice (39). The release of cyclooxygenase-dependent vasoconstrictor factors in the arteries of the mesenteric region is more significant in spontaneous hypertensive rats (SHR) in male gender but not in females (36). The coronaries of male rats are more sensitive to the vasoconstrictor effects of endothelin-1 than coronaries of females (37). Myogenic tone of coronary vessels and vasoconstrictor response to thromboxane agonists were also more pronounced in male rats (38). In contrast, based on our previous research, there is no difference in the Phenylephrine (Phe)-induced vasoconstriction of renal arteries between the sexes (128). In our present research, the carotid artery contraction induced by phenylephrine is higher in male rats than in females, irrespectively from vitamin D status.

The risk and severity of stroke are clearly correlated with serum vitamin D levels (200, 201). An increase in arterial stiffness in vitamin D deficiency may be a crucial factor in the increased cardiovascular risk. Vitamin D supplementation may reduce this effect in both sexes (104). The reduction in arterial stiffness due to vitamin D supplementation may be dose-dependent, as seen in a vitamin D deficient overweight human study (202). Vitamin D deficiency may also be a factor in the enhanced arteriosclerotic process affecting the carotid arteries (203). The strength of Phenylephrine-induced contraction in our studies was shown to be increased in both sexes in vitamin D deficient conditions. The weakest contractile response to phenylephrine developed on the female's vitamin D added rat's carotid ring. The strongest contractile response was seen in male rats with deficiency of vitamin D. The contraction capacity of vitamin D deficient females has reached that of vitamin D supplemented males. Studies consistent with the results we have described are also available for mesenteric arteries, where vitamin D deficiency doubles the myogenic tone of these vessel (204).

Based on the previous results of our research group and the literature data, we can state that vitamin D deficiency and supplementation have different effects on contractility, both for different vessels and for different sexes. Examination of coronary arteries in vitamin D deficient diets reduced the contractile effects of thromboxane A₂ (125). However, the renal artery in female rats showed increased contraction with phenylephrine, but this was not observed in male animals (128, 129). Vitamin D inhibits TP expression (205). A histological change in vitamin D deficient animals showing increased thromboxane A₂ receptor (TP) staining in the carotid wall of MVD- animals may be due to the cessation of TP receptor depletion inhibition due to vitamin D deficiency. The presence of estrogen may reduce TP receptor expression (206), which may play a role in the absence of a significant increase in TP signal intensity in the carotid artery in FVD- rats. According to our data, the effect of prostanoids in the carotid arteries of female animals resulted in contraction, as the general COX inhibition used in our study leads to a decrease in Phe-induced effect. This effect does not occur in vitamin D deficient conditions. In our study, the simultaneous inhibition of the COX is enhanced the contractile force just in male vitamin D deficient animals. These observations have not been published earlier by other authors.

In our study, the observed histological alterations may also explain the differences in vasoconstriction results in different study groups.

The staining of both elastic fibers and smooth muscle cells is markedly affected by sex and the serum vitamin D level. The number of elastic fibers in the LAD coronary arteries is apparently less in the male study group than in females (168). Previous data have shown that vitamin D deficiency has a negative effect on the number of the elastic elements of the aorta (207), while this effect is not observed in other vessels, such as cerebral vessels (41). The carotid wall of FVD+ animals contain significantly more elastic fibers than in rats from all other study groups. Thus, it can be stated that the effect of vitamin D on the elastic structure of blood vessels does not show only sex differences but differs with respect to other and other blood vessels. In our study, immunostaining in MVD- rats smooth muscle actin was found to be the weakest of all groups. In the same direction, vitamin D has previously increased SMA levels in the aortic wall of rats on a high fat dietary circumstance (208), which may affect and alter the change observed of SMA density.

Our observations forming the present Dissertation unanimously prove the effect of vitamin D deficiency and supplementation on different parts of the vascular system. The observed coronary network geometry changes, alterations in carotid contractility and relaxation as well as a histological remodeling of the artery wall while do not show explicit pathological deterioration, their direction can be supportive for pathological changes induced by more powerful noxa. Also, we first described that the vascular effects initiated are dependent on sex.

When drawing conclusions from the results of our experiments, we must also address the limitations of our investigations. We worked with a small number of animals during both the coronary mapping and the contractility investigations of the carotid arteries. The clear reason for this was the complexity of the methodology, nature of the investigations, and the complexity of the statistical analysis. At the same time, the inclusion of more animals in the studies would have either made the process very long or required the participation of additional researchers in the study. However, this would have increased the interobserver variability, especially during the analysis of coronaries.

Another limitation of our studies is the eternal dilemma of whether conclusions can be drawn for the human population based on information obtained from animal models. We were able to mitigate both mentioned limitations somewhat by standardizing the test conditions and the usage of homogenous genetic properties of the animals.

5. CONCLUSIONS

Our experiments focused on the following questions:

1) Are dietary alterations of vitamin D and the results serum vitamin D alterations can change the formation of the complex geometry of the left anterior descending coronary artery (LAD) network in rodent model? Do the branching and segments property change? Does the distribution of cylindrical ring units of arteries of different diameters show alteration?

Although there are no network abnormalities or difference of the number of segments found, several alterations of the LAD system occurred associated with serum vitamin D levels. An increase in the wall thickness of larger segments, a decrease in the number of smaller-diameter ring elements, and changes in bifurcation angles in different study groups indicate some involvement of vitamin D in the morphological development of coronary artery networks. Thus, due to the richer network created by vitamin D supplementation may improves tissue perfusion. In the vitamin D supplemented animals, the networks were richer, the branching pattern was optimal, with no abnormalities. While these can only be expected to have moderate hemodynamic effects, they can alter pathological processes.

2) Are there any sex-specific differences in the contractility of the carotid artery associated with different serum vitamin D levels?

Low vitamin D levels such as male gender caused enhanced contractile properties of carotid arteries of the rats. The shifting balance of circulating plasma prostanoid levels increases the effect of vasoconstriction in male vitamin D deficient rats in the presence of L-NAME. This can be a result of the marked increase of TP immunostaining of this group. COX inhibition alone leads to a decrease in Phe-induced contraction in vitamin D supplemented female rats, which cannot be seen in vitamin D deficiency. Although does not affect the contraction of the carotid arteries of the male rats.

3) If there are, are these effects comes with related sex-specific alterations of the histological structure of the carotid arteries of the rat?

Changes of the contractility of the carotid arteries induced by vitamin D alterations apply together with sex-specific histological alterations. Staining of elastic fibers became

fainter because of the vitamin D deficiency in female animals, but not in male rats. Smooth muscle actin staining increased, the thromboxane receptor staining showed a marked increase in the male vitamin D deficient group. Decreased nitrate stress level based on the histology remained lower in both male groups regardless from VD supply.

Literature data clearly indicate a sex-dependent involvement of vitamin D in cardiovascular risk. In connection with coronaria mapping, we shed light on a new role of vitamin D in the process which has not been described so far. Our results described during the histological and contractile examinations of the carotid arteries may contribute to a clearer understanding of these gender differences of the already known clinical observations.

6. SUMMARY

Vitamin D deficiency alters several cardiovascular properties and increases the risk of pathological events. The aim of our research was to identify whether alterations in vitamin D diet can change the coronary resistance artery system geometry and the contractile function as well as the histological construction of the carotid artery. Further, is there any sex specific difference in the response of the carotid arteries in this respect. Low serum levels of vitamin D were reached by dietary changes. One of the groups of randomly divided animals got vitamin D free diet, while animals of the other experimental group have been offered standard laboratory diet and received additional vitamin D. After 8 weeks, analysis of the LAD coronary artery network has been made by video microscopy technique. Dietary changes did not affect neither the number of morphological abnormalities, nor the number of segments. Smaller lumen surface area and lengthier 1-4 order branches were found in the deficient group. Vitamin supplemented rats had richer coronary resistance artery networks. An increase in the wall thickness of larger segments, a decrease in the number of smaller-diameter ring elements, and changes in bifurcation angles in different study groups indicate some involvement of vitamin D in the morphological development of coronary artery networks. For the analysis of the carotid arteries, male and female Wistar rats were randomly selected into male vitamin D deficient, male vitamin D supplemented, female vitamin D deficient and female vitamin D supplemented group. Both gender groups were divided into subgroups according to the dietary method mentioned above. The isolated carotid artery segments contractile abilities were examined by wire myography. As a result, both vitamin D deficiency and male sex lead to increased Phe-induced contraction. Blocking of prostanoid signaling by indomethacin resulted in decreased contractile capacity in females. Staining of elastic fibers decreased by vitamin D deficient female rats, but not in males. SMA levels were significantly lowered, but the thromboxane receptor was elevated in vitamin D deficient males. Decreased nitrate stress was detected in both male groups independently from vitamin D supply. These findings may contribute to vitamin D dependent changes in cardiovascular mortality and may bring closer how the interactions between vitamin D deficiency and sex can play a role in the sex differences of cardiovascular risk.

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Article

Vitamin-D Deficiency and Supplementation Altered the Network of the Coronary Arteries in a Rodent Model—In Situ Video Microscopic Technique

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Abstract: The aim of our study was to identify whether vitamin-D deficiency (VDD) can alter the geometry of the coronary-resistance-artery system. Male Wistar rats were divided into vitamin-D-deficient (VD−, n = 10) and vitamin-D-supplemented (VD+, n = 8) groups. After eight weeks, branches and segments of the left-anterior-descending-coronary-artery (LAD) network were analyzed by a video-microscopy technique. Segments were divided into 50 μm-long cylindrical ring units. VDD did not increase the number of morphological abnormalities. The number of segments did not differ between the groups (VD−: 210 and VD+: 224; pooled data of 8 networks). A larger lumen area of branches was found in VD+ group, while 1–4-order branches were lengthier in the VD− group. VD− rats had less rich coronary-resistance-artery networks in terms of 50 μm-long units. (VD−: 6365 vs. VD+: 6602; pooled data of 8 networks). VD+ animals were richer in the 100–350 μm outer diameter range, and VD− animals were richer in the 400–550 μm-diameter units. In VD− rats, 150–200 and 300 μm units were almost missing at higher flow distances from the orifice. Serum vitamin-D alterations caused by dietary changes can affect the geometry of the coronary-artery network, which may contribute to vitamin-D-dependent changes in cardiovascular mortality.

Keywords: vitamin-D deficiency; network; left-anterior-descending coronary arteries; LAD; cardiovascular disease; video-microscopic technique

1. Introduction

It has been a hundred years since McCollum first used the term ‘vitamin D’ in 1922 [1]. Since then, vitamin D has been known as a regulator and key molecule of calcium metabolism, serum calcium levels, and bone mineralization in the human body [2–5]. Beyond its skeletal effects, the role of vitamin D has been confirmed in numerous different biochemical processes and diseases including but not limited to mental and psychological disorders [6], cancer [7], immunological aspects [8], as well as pregnancy and neonatal outcomes [9]. Therefore, some consider it as a general physiological regulatory molecule.

Its role in the cardiovascular system is being extensively studied. Observational studies have confirmed the linkage of vitamin-D deficiency with hypertension and cardiovascular-related deaths [10,11]. As an explanation for this, the vitamin-D receptor (VDR) has been

found to be widely distributed in the cardiovascular-system cells. However, the role of vitamin-D supplementation in reducing cardiovascular harm is unclear based on the results of comprehensive studies. Most comparative studies leave the question open [12–19]. However, there are studies that confirm [20] and some that do not support [21,22] the role of vitamin-D supplementation in reducing the cardiovascular mortality rate.

One of the most studied cardiovascular consequences of vitamin-D deficiency is its role in myocardial ischemia [23]. There are several mechanisms that can contribute to this, all of which can be affected by vitamin-D deficiency. Most studies have focused on the effect of vitamin D on different cell types (e.g., cardiomyocytes or vascular smooth-muscle cells) [14,24]. Arterial stiffness [25], altered endothelial function [26,27], increased atherogenesis [28], and changes in oxidative-stress tolerance [29] can all play a role in it. The topic of altered vascular contractility and relaxational ability associated with vitamin-D deficiency was studied by our research team in a rodent model in coronary arteries [30,31], cerebral arteries [32,33], renal arteries [34], carotid arteries [35] and the aorta [36].

The examination of the geometry of the vascular network, the description of the hemodynamically advantageous course, and branches of the vessels can be traced back to the rules described by Murray [37,38]. Although it has been known for almost one hundred years that deviations from this law reduce the efficiency of circulation, most of the research on vascular examination has focused on the histology, biochemical properties, and contractility of blood vessels. The development of the micropreparation technique of intramural coronary-resistance-artery networks in the rodent model [39,40] and the increasingly widely used technique of video microscopy have made it possible to study the vascular-network system in its complexity and to observe the effect of different variables on the whole network. Our research team successfully demonstrated the effect of hypertension, aging, and training on the entire geometry of the coronary network [39,41,42] using this technique.

In our study we sought the answer to how vitamin-D deficiency and the biochemical processes influenced by it can change the geometry, branching, and distribution of arteries of different diameters of the left-anterior-descending-artery (LAD) network. Our hypotheses were: (1) vitamin D does not only affect the histological structure and contractile function of the coronaries, but also plays a role in forming the geometry of the entire network, thus gaining a potential role in cardiovascular mortality; (2) the lack of vitamin D, which is known to be involved in angiogenesis, may produce hemodynamically disadvantageous network anomalies in the coronary network; (3) that changes may occur in the location of the vascular-network unit population due to vitamin-D deficiency and supplementation.

2. Materials and Methods

2.1. Ethical Approval and Animals

The study was designed and performed based on the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011) and the European Union (Directive No. 2010/63/EU). All procedures were approved by the Ethical Committee of Hungary for Animal Experimentation and University authorities (permission number: IRB: 8/2014 PEI/001/1548-3/2014, PEI/001/820-2/2015). Four-week-old male ($n = 18$) Wistar rats (Simmelweis University in agreement with Charles River Ltd., AnimaLab, Vác, Hungary) were randomly divided into two experimental groups: a group with vitamin-D deficiency (VD⁻, $n = 10$) and a group with vitamin-D supplementation (VD⁺, $n = 8$).

2.2. Chemicals

Vigantol oil (20,000 IU/mL cholecalciferol suspension) was provided by Merck/Merck Serono (Darmstadt, Germany). For ex vivo video-microscopic analysis, specimens were immersed in normal Krebs–Ringer solution (nKR), which consisted of the substances

published in [41]. The substances used for the nKR were obtained from Reanal (Budapest, Hungary) and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA–Budapest, Hungary).

2.3. Chronic Treatment of the Rats

During the 8-week-long chronic-treatment period, rats were housed in constant environmental conditions (relative humidity (40–70%), constant room temperature ($22\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$) and light–dark cycle (12 h each)). The animals were provided with different laboratory rat chow (with different composition, see later) and tap water ad libitum according to the following group protocols. Vitamin deficiency was induced by a vitamin-D-free diet ($<5\text{ IU/kg}$ Vitamin D₃, Vitamin D Free Lab Rat/Mouse Chow, Ssniff Spezialdiäten GmbH, Soest, Germany) for 8 weeks (the average 25-OH-D₃ level at the end of chronic treatment: $3.59 \pm 0.21\text{ ng/mL}$) [32,43]. Animals of the VD+ group were fed a standard laboratory diet (1000 IU/kg of Vitamin D) for 8 weeks. Oral administration (through a gavage cannula) of additional vitamin D was given as follows: 500IU cholecalciferol on week 2, and weeks 4–8, a weekly dose of 140 IU/100 g (the average 25-OH-D₃ level at the end of chronic treatment: $19.66 \pm 0.81\text{ ng/mL}$) [32,43]. No unexpected medical condition, complication or side effect was observed during the treatment period.

2.4. Preparation and Recording of LAD Coronary Networks

After chronic treatment, the preparation of coronary-resistance-artery networks from the heart and in situ video-microscopy recording during perfusion were performed as previously described [40]. In brief, after anesthesia (Nembutal, 45 mg per kg, intraperitoneal), the heart was removed and the LAD coronary-artery network was prepared by careful microdissection in cold Krebs–Ringer solution under high magnification [40]. With this technique, the segments of the LAD remained intact and branches larger than 80 μm became visible. After cannulation of the orifice, the network of the LAD was perfused with nKR solution (pH = 7.4, 37 $^{\circ}\text{C}$, bubbled with O₂ 20%, CO₂ 5% and N₂ 75%) at close to in vivo pressures. After a few minutes of equilibration, the coronary network was measured by a video microscope using different magnifications (low and high magnifications, 8.58 and 1.47 $\mu\text{m}/\text{pixel}$). For accurate geometric reconstructions of the networks, low- and high-magnification images were photographed and then analyzed off-line (ImageJ software, NIH, Bethesda, MA, USA) as previously described [39]. The pixel μm calibration was performed using a micrometer etalon (Wild, Heerbrugg, Switzerland).

2.5. The Coordinate System and Geometric Analysis

Good-quality low- and high-magnification pictures taken from perpendicular position were selected to rebuild a horizontally stretched network for analysis. All bifurcations and segments of the coronary network were then marked in the $>80\text{ }\mu\text{m}$ range. A coordinate system was created based on the high-magnification pictures of the networks as previously described [39]. In brief, the X-axis was created between the orifice and the apex of the heart. The Y-axis was erected perpendicular to the X-axis, with positive values in the direction of the left ventricle. The zero point for both axes was the orifice. Segment lengths and bifurcation angles were measured, images of segments were divided into 50-micrometer-long cylindrical units, and the diameter, direction and coordinate position of all components were determined as shown in Figure 1.

2.6. Segment Analysis

The whole network was divided into segments at bifurcations and the segments were then numbered. Although the diameter of the vessel generally does not change along the segment, outer and inner vessel diameters were also measured at three points along the segmental axis. Distances of the branching points from the origin, angles of the segmental axes of two related segments, and angles with the coordinate were also analyzed. For each network component (bifurcations, segments, ring units), the direct distance from the orifice was computed using the coordinates. In addition, for each segment, a length for

the potentially curved axis was also computed. This way “flow distances” from the orifice or for the segments could be compared with direct distances, giving an opportunity to calculate the tortuosity of the network. Segmental analysis was performed by counting the number of segments and measuring their length, as well as the outer diameter and wall thickness. From the inner-radius (r_i) data, the lumen cross-section area was calculated according to the following formula: Lumen cross-section area (μm^2) = $r_i^2 \times \Pi$.

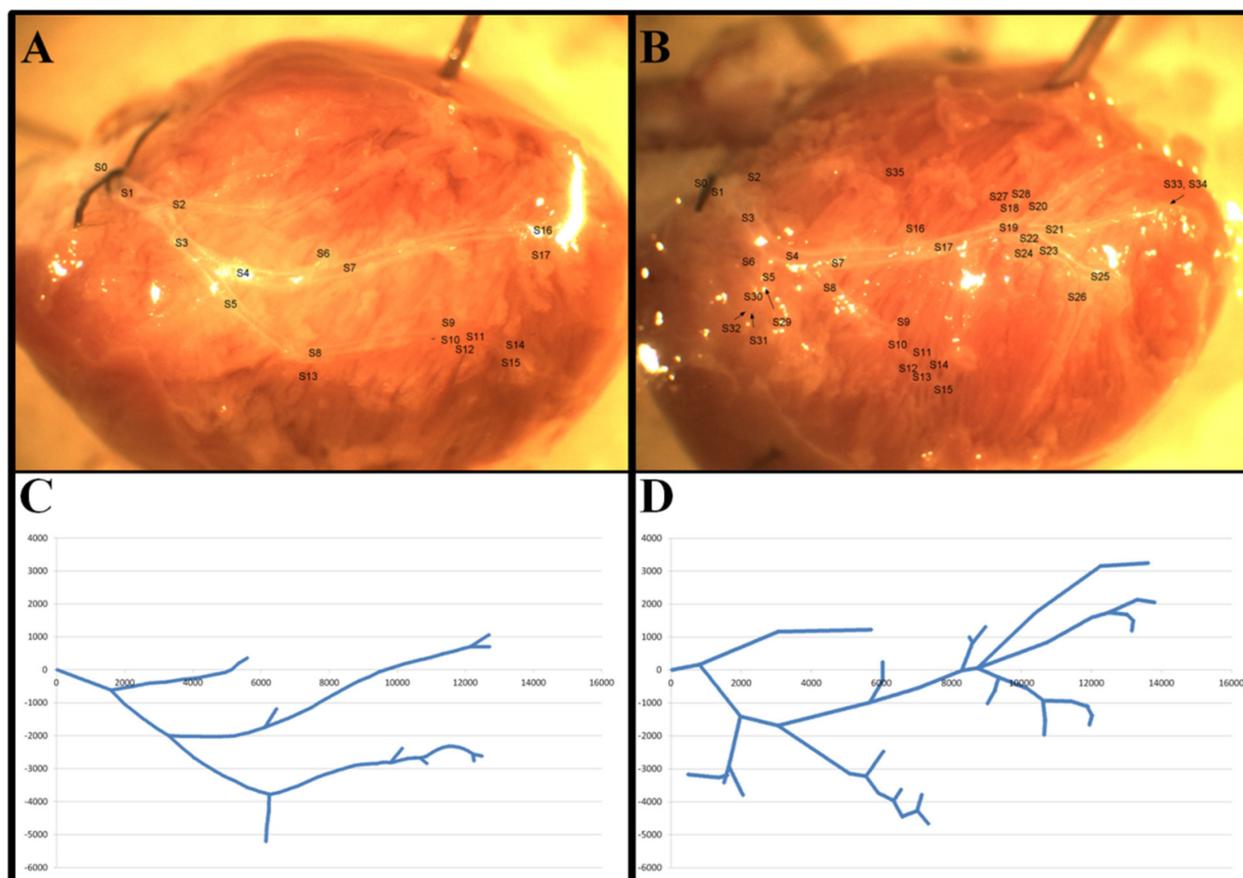


Figure 1. Representative video-microscopic images of segmental and branching analysis (A,B) and mapping of the LAD networks in a coordinate system, following 50 μm -ring-unit analysis (C,D). Note the difference between the two experimental groups in the network density of the LAD coronary artery, shown in the image and the associated coordinate system of a typical vitamin-D-deficient (A,C) and a typical vitamin-D-supplemented animal (B,D).

2.7. Branching Analysis

All branches were identified and analyzed as previously described [39]. All bifurcations were sorted into dichotomic, multiplex or lateral branching categories. All bifurcations were tested for the validity of Murray’s law: $D_{om}^3 = D_{od1}^3 + D_{od2}^3$, where D_o is the outer diameter in μm , and m , d_1 and d_2 are the mother and daughter branches, respectively. The asymmetry index (A_i) was calculated according to the following formula: $A_i = D_{od1}/D_{od2}$, where D_o is the outer diameter in μm , and d_1 and d_2 are the daughter branches (the data of the larger daughter branch were always placed in the numerator/top).

2.8. Analysis of 50 μm -Long Vascular Ring Units

Theoretically, all coronary-artery networks were divided into 50 μm -long ring units as a base unit of the network as previously described [39]. The ring units were located in the X–Y coordinate system. The outer and inner diameters, wall thickness, X and Y coordinates for the ring-unit center, angle of axis with the X-axis, flow distance, and the direct distance

from the orifice were measured. The ring-unit analysis was performed by constructing lists of ring units in a certain inner/outer-diameter range and at a certain direct/flow distance from the orifice.

2.9. Network Anomalies

In addition to the measurable parameters of the networks, other hemodynamically significant alterations were also recorded. Parallel-running branches, broken courses, and multiple branching (e.g., triple) present in the systems were counted. Tortuosity was measured. Tortuosity (T), curvature, and ratios of segments were computed by comparing the direct distance between the start and end points of the segment as well as the potentially curved length of the segment's axis following the route of blood flow: $T(\%) = 100 - (\text{segment length in the airline } (\mu\text{m}) \times 100 / \text{segment length in real } (\mu\text{m}))$.

2.10. Statistical Analysis

GraphPad Prism 5, SPSS Sigma Stat and Excel software were used for statistical analysis. All data are presented as mean \pm SEM. In the case of normal distribution (tested using the Shapiro–Wilk method), the two-tailed unpaired Student's *t*-test was performed. The Mann–Whitney test was performed in the case of non-normal distribution (in case of 'Flow lengths of the segment' and 'Wall thickness of 50 μm ring units as a function of different diameter ranges'). Morphological abnormalities were counted, pooled and normalized in 8 rats for all perfused networks. Their number was determined by the test. Frequencies of ring units in different diameter ranges in VD+ and VD− coronary networks were compared with the χ^2 test. The Pearson correlation method was used to evaluate the interconnection between bifurcation asymmetry and angle. A 3D scatter plot was used to show differences in bifurcation branch angle as a function of vessel diameter. The level of deviation of the flow route from the direct distance from orifice and the diameter of the ring were analyzed on 3D plots of two-dimensional histograms. The number of ring units in a given diameter and flow-distance range was analyzed in two-dimensional histograms and visualized in 3D (color-coded) maps. $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Body-Weight, Heart-Weight and Blood-Pressure Data

There was no significant difference between the VD− and VD+ groups in terms of body weight (VD−: 481 ± 15 vs. VD+: 477 ± 19 g) or heart weight (VD−: 1.37 ± 0.04 vs. VD+: 1.32 ± 0.08 g). The measurements of the mean blood pressure through cannulation of the right carotid artery showed no difference between the two groups (VD−: 95.39 ± 4.35 vs. VD+: 88.18 ± 6.57 mmHg, animals in Nembutal anesthesia).

3.2. Segment Analysis

The number of coronary-resistance-artery segments in the subsurface network, down to an outer diameter of 80 micrometers, did not differ between the two groups (VD−: 210 and VD+: 224 segments; pooled, normalized data of 8 networks). One characteristic difference was the significantly larger lumen area of the main (first-order) branches in VD+ group. The lumen diameters did not differ during the following branching steps (Figure 2A). Additionally, 1–4-order branches were lengthier in VD− rats (significant with the paired *t*-test for 1st- and 4th-order branches, (Figure 2B)). It is important to note that 11–12-order branches were found only in the VD+ group in the subsurface network (Figure 2).

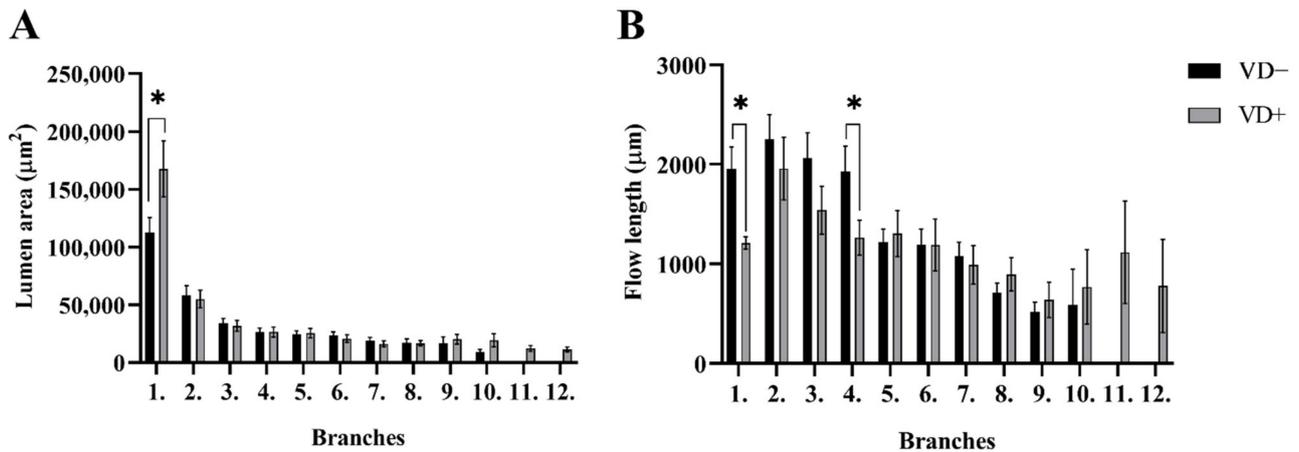


Figure 2. Segments analysis. (A) Lumen area of the segments from the VD− and VD+ animals. The lumen area of the first-order branches was significantly larger in VD+ group. (B) Flow lengths of the segments from the VD− and VD+ animals. The 1st- and 4th-order branches were lengthier in VD− rats. Values are means ± SEM. Two-tailed unpaired Student’s *t*-test and Mann–Whitney-test. * *p* < 0.05 VD− vs. VD+.

3.3. Branching Analysis

One characteristic of microvascular bifurcation geometry is that larger daughter branches tend to deviate less from the axis of the mother branch than smaller daughter branches. As a result, the branch angle between daughter branches increases with the increasing asymmetry index. In substituted networks it is duly seen (Figure 3A; significant with the Pearson correlation). Such a linkage is clearly missing in the vitamin-D deficient animals (Figure 3A).

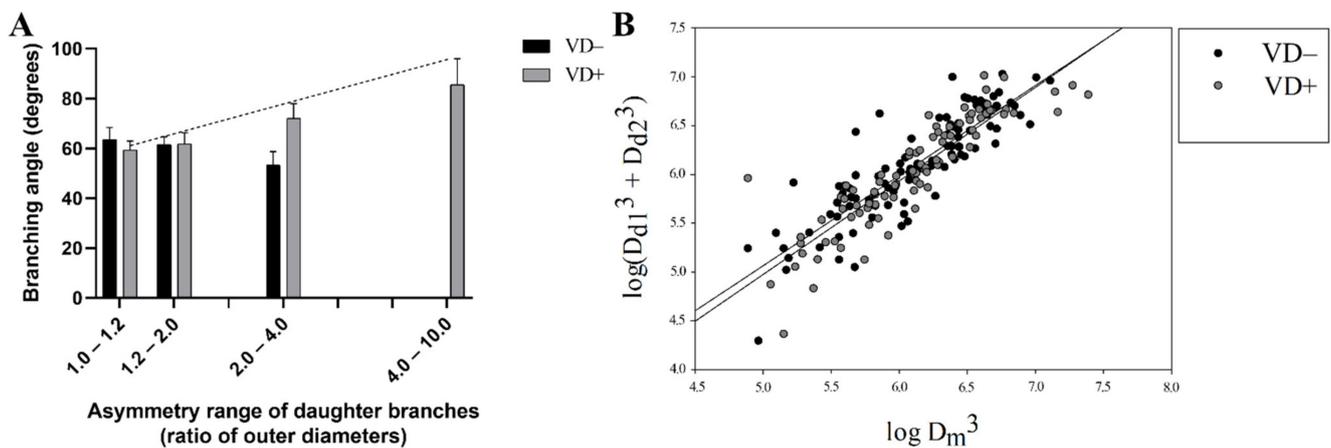


Figure 3. Analysis of branching. (A) Asymmetry range of daughter branches (ratio of outer diameters). The asymmetry index (with increasing branching angle) was significantly elevated only in VD+ groups (with Pearson correlation, *p* < 0.05). (B) The Murray law. Branches are obeying the Murray law.

Another characteristic of microvascular bifurcation geometry is that the lumen diameters of daughter branches obey the Murray law. Figure 3B demonstrates that bifurcations of both groups fairly adhered the Murray law (scatter from the expected X = Y line was almost equal, n.s. with the F probe).

3.4. Abnormalities

Vitamin-D deficiency did not elevate the number of such morphological network abnormalities as parallel running, broken courses, multiple branching, or tortuosity (Table 1).

Table 1. Pooled number (normalized in 8/8 rats) of morphological deformities found in the resistance-artery network of the left-anterior-descendent coronary artery of vitamin-D-deficient (VD−) and vitamin-D-supplemented (VD+) groups.

Morphologic Deformity	VD−	VD+	Chi2 (χ^2) Probe Significance Level
Parallel running	2	3	0.65
Broken course	7	6	0.74
Multiple branching	11	8	0.49
Tortuosity > 8	7	5	0.56
Sum of all deformities	27	22	0.48

3.5. Vascular Ring-Unit Analysis

VD− animals had somewhat less rich coronary-resistance-artery networks than VD+ rats. (Figure 1) When the whole network was divided into 50 μm -long units, they had a significantly lower number of such units (6365 vs. 6602; pooled, normalized data of 8 animals; $p < 0.0374$ with the χ^2 probe). Figure 4A demonstrates that this elevated number of vascular units in VD+ animals was present throughout the 100–300 μm outer diameter range. The only exception is at 250 μm , the maximum of the histogram in VD− animals pushed upward from the 200 μm of the substituted animals.

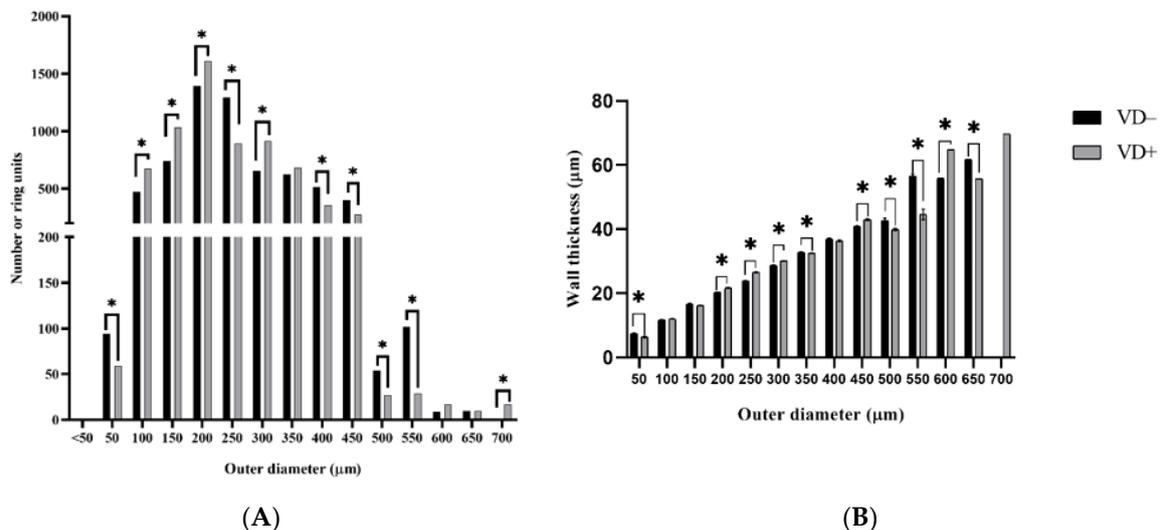


Figure 4. Analysis of ring units. (A) Number of 50 μm ring units as a function of different diameter ranges. In VD− networks, the number of rings decreased in the range of 100–300 μm (except at 250 μm), and the number of rings increased in the range of 400–550 μm . Normalized in 8/8 rats. Significantly different with the Chi-probe ($p < 0.05$). (B) Wall thickness of 50 μm ring units as a function of different diameter ranges. Wall thickness was increased in the 50, 350, 500, 550 and 650 μm range in VD− group. However, the wall thickness was bigger in the 200, 250, 300, 450 and 600 μm range in VD+ group. Values are means \pm SEM. Mann–Whitney-test. * $p < 0.05$ VD− vs. VD+.

However, VD− animals were richer in larger-diameter units (400–550 μm). In practically the same large-diameter group, a thickening of the wall was also demonstrated with an opposing alteration in the most frequent 200–300 μm diameter range (Figure 4B).

The next question is at what location of the network such vascular unit-population changes do occur. The two-dimensional histograms of Figure 5 demonstrate that in vitamin-D-deficient rats, a new population of 250 μm units appears at a 6–9 mm flow distance from the orifice, while at the same locations there is a diminishment of 350 μm units. In vitamin-D-deficient rats, 150–200 and 300 μm units are almost missing at 10–15 mm flow distances.

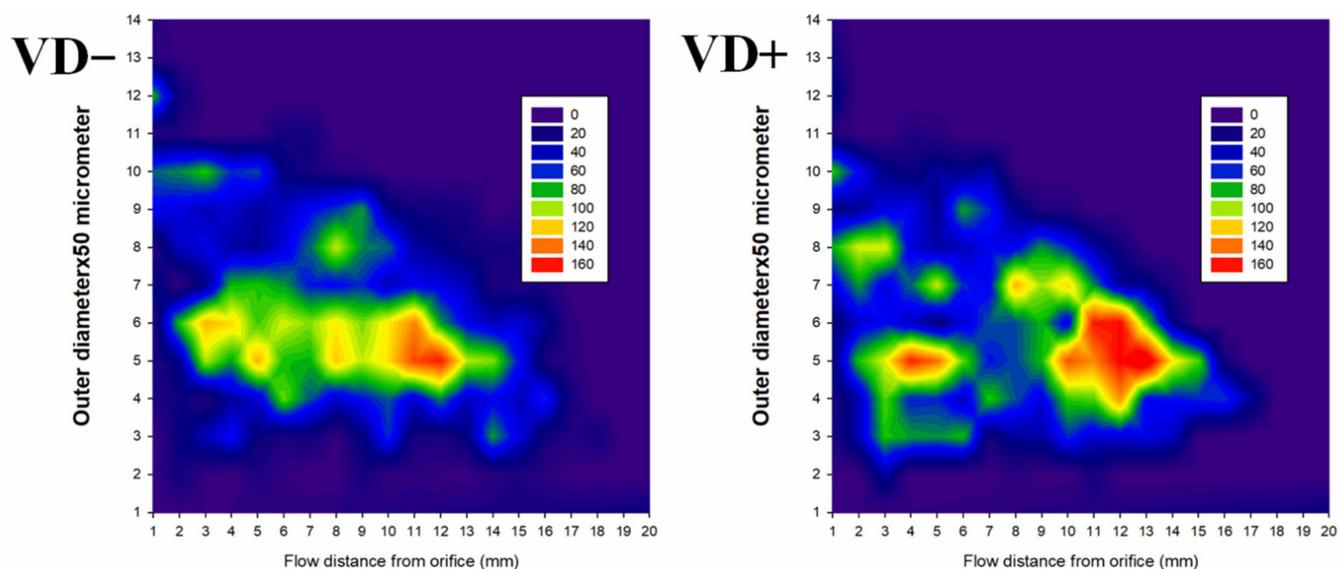


Figure 5. Frequency of ring units (color-coded) for different diameters and flow distances from the orifice. Note that in VD– rats a new population of 250 μm units appears at a 6–9 mm flow distance from the orifice, while at the same locations there is a diminishment of 350 μm units. In VD– rats, 150–200 and 300 μm units are almost missing at 10–15 mm flow distances.

4. Discussion

Our first studies on the whole network geometry of in situ perfused coronary-artery networks in Vitamin-D-deficient and substituted animals demonstrated that chronic vitamin-D deficiency induces characteristic changes in network geometry. In the present study, we first demonstrated that vitamin-D deficiency affects cardiac-tissue perfusion without abnormalities in coronary branching patterns that modulate cardiac hemodynamics. The major finding of our investigation can be summarized as follows: (1) VDD did not increase the number of morphological abnormalities; (2) VDD resulted in a less rich coronary-resistance-artery network; (3) VDD resulted in 150–200 and 300 μm units that were missing at higher flow distances from the orifice; (4) in contrast, vitamin-D supplementation resulted in a richer network; (5) vitamin-D supplementation resulted in a larger lumen area of the branches, the branching pattern was optimal, and abnormalities did not increase.

It is a known fact that vitamin-D deficiency increases the risk of cardiovascular events, although vitamin-D supplementation does not clearly balance this effect, as demonstrated by several meta-analyses [10–12,14,17,18,20–22]. Vitamin-D-deficiency-induced cardiovascular risk is associated with a combination of several factors [44]. Low vitamin D has been shown to be associated with high blood pressure [45,46] obesity [47–50], insulin resistance [51,52], and dyslipidemia [53]. In several studies, a description of normal 25-OH-D3 levels can be found. Trechsel et al. published values of 40 nmol/L (16 ng/mL) in animals consuming a normal vitamin-D diet [54]. At the recommended daily intake (values projected onto human model: 0.015 mg/day, which corresponds 300 E/day), Mirhosseini et al. achieved vitamin-D levels of 17.2 ng/mL in Wistar rats over four weeks of treatment.

According to the same article, at a high daily dose of vitamin D (values projected onto human model: 0.25 mg/day, which corresponds 5000 E/day), vitamin-D levels of 43.2 ng/mL were achieved in Wistar rats over four weeks of treatment, while a vitamin-

D-free diet resulted in 12 ng/mL [55]. Wilson et al. reported levels of 37–38 ng/mL of 25-OH-D3 but also used vitamin-D supplementation in addition to the standard diet [56]. These values were not approached in any of the groups.

For extremely low ranges, Treschel considered a vitamin-D deficiency below 25 nmol/L (10 ng/mL) [54], which we have clearly achieved in our animal model. The definitively toxic concentration is 360 ng/mL based on Takács et al. [57] and 224 ng/mL according to Mirhosseini et al. [55]. Such toxic values were not achieved in any of the groups of our study.

According to Halloran's work, vitamin-D levels of 14-day-old breastfed Holtzman rats whose mother received 25U daily vitamin-D supplementation was 10 ng/mL. A vitamin-D level of 8 ng/mL was found after weaning at the age of 25 days, and 9 ng/mL was found in animals three weeks after being weaned from breast milk and fed with normal laboratory formula during this period [58]. Vitamin-D levels in Holtzman rats that had just been weaned in Weishaar's work were below the measurement range and remained there after a nine-week vitamin-D-free diet, while it increased to 9.5 ng/mL after six weeks and 14.1 ng/mL after nine weeks with vitamin-D supplementation of 30 E/day [59].

Thus, we can suspect that the rat weaned from milk suffers from a relatively moderate vitamin-D deficiency, which we were able to normalize and even slightly exceed the normal level in our model by supplementation. On the other hand, by our model an eight-week vitamin-D-free diet starting from weaning kept vitamin-D levels persistently low. The effects of vitamin-D deficiency on the histological characteristics of vascular cross-sections and the contraction and relaxation properties of coronaries [30,31] has already been demonstrated by our research group.

We found no difference between the body weight of the study groups. Vitamin D also affects the differentiation of myocardial cells and affects myocyte proliferation through its action on myoproliferative genes and the renin–angiotensin system [60–62]. However, we found no difference in heart weight between the two groups, making it easier to compare the coronary networks.

For the technical implementation of our study, we chose the micropreparation and video-microscopic analysis of the LAD branch system [39–42]. The technique allows the whole network to be analyzed in its complexity, using physiological pressure conditions to which the blood vessels respond with their own myogenic tone. This contributes to a proper evaluation of the dimensions of the specific parts of the network involved. The spatial formation of the coronary network ensuring that all parts of the heart muscle tissue receive equal and adequate amounts of oxygen and nutrients. This process is regulated by many factors [63]. The network must be of adequate resistance and hemodynamically advantageous to distribute blood flow to the highly demanding ventricular tissue. The length, the number of segments, geometry and quantity of branches, bifurcations, their potential tortuosity, diameter of the lumen, thickness of the wall, and the distribution of all these parameters will, be among others, a function of the distance from the origin of the network (in that case the coronary orifice). Knowing the effect of vitamin D in the regulation of vascular remodeling [56,57], we could expect that there is a difference between the two groups in the appearance and geometric characteristics of the network as a whole system. A geometric analysis of the network allows us to recognize different anomalies and to analyze the systemic effects of vitamin-D deficiency on vascular-network formation, vascular cross-sectional structure, and changes in the coronary network, possibly contributing to increased cardiovascular mortality.

Basic characteristics of the morphometry of a coronary network and the effect of hypertension have been studied in different models [64–67]. Based on the work of Murray and Zamir, we can gain insight into the properties of hemodynamically beneficial vascular networks [37,38,68]. Accordingly, we considered multiple branches, branches running in parallel, broken courses, and a high range of tortuosity as disadvantageous patterns requiring higher-than-optimal mechanical energy. Based on previous work by our research group, we have demonstrated that high blood pressure increases the incidence of these

anomalies in the rat LAD network. [42] In earlier works, we have found that the Murray law of bifurcations was maintained in cases of hypertension, aging and physical exercise [39,41]. In our present study, there was no significant difference between the two groups in terms of the unfavorable coronary anomalies characterizing aged and hypertensive networks [42]. At the same time in our study, we found no difference between the mean blood pressure of vitamin-D-deficient and supplemented animals. All this suggests that although vitamin D is known to play a role in vasculogenic processes [69,70], it is not vitamin-D deficiency alone that causes vascular-network anomalies, but in the long term it may be caused by high blood pressure due to vitamin-D deficiency. In Weishaar's previous study [59], calcium, creatin phosphokinase (CPK) and phosphate levels along with blood pressure were examined in young rats as a function of vitamin-D supplementation. It was shown that vitamin-D deficiency significantly increased the blood pressure of the animals between weeks two and six of the diet; however, there was no difference in blood pressure from week seven to nine of the diet. Consistent results were obtained in our present study after eight weeks of treatment. In Weishaar's study [59], perturbed Ca homeostasis was suggested to be responsible for the very early rise in blood pressure; however, the eight-week diet had no effect on it. On the other hand, recent reports stress the relationship between long-term vitamin-D deficiency and hypertension [10,11,14,45,46]. The atherogenic effect of the vitamin-D deficiency may be responsible for the development of hypertension in the long term [69,70]. According to the rheological principles of Poiseuille, in a hemodynamic system, the length of the vessels is directly proportional, while the fourth power of the lumen diameter and the number of branches connected in parallel are inversely proportional to the resistance of the network. We can suppose that in the vitamin-D-deficient experimental group, the vascular resistance of the coronary arteries might be higher. In our experimental model, coronary-network abnormalities that increased network resistance due to vitamin-D deficiency were expected to result in a long-term increase in blood pressure, ensuring an adequate supply of nutrients to the tissues.

Further analyzing our coronary-artery networks with a focus on branching, we found that more asymmetric bifurcations do not have larger bifurcation angles in vitamin-D-deficient rats as previously expected and as shown in the VD+ group. This can be considered a disadvantageous alteration from the hemodynamic point of view. Vitamin-D deficiency, however, did not affect the regulation of the lumen diameter by shear forces, and the Murray law was strictly maintained in both groups.

Analyzing the coronary-resistance-artery segments in the subsurface network, the number of segments with outer diameters down to 80 micrometers did not differ between the two groups. At the same time, some characteristic difference was found. A significantly larger lumen area of the main (first-order) branches in the VD+ group may allow for greater blood flow. Lumen diameters did not differ during the following branching steps. Additionally, 1–4-order branches were lengthier, and segments formed by them were longer and had thicker walls with reduced cross sections in VD– rats, which may result in greater resistance of those specific segments. It is important to note that 11–12-order branches were found only in the VD+ group. This result objectively confirmed the preliminary observation made during the review of the coronary networks that the LAD networks of the vitamin-D-deficient group were much denser. In further analysis of the network, the coronary network was divided into 50 μm elements and the number of ring elements, diameter, wall thickness, lumen cross section, and the distribution of these ring elements of different thicknesses as a function of distance from the orifice were examined. Although heart masses did not differ, the number of normalized ring elements of the network was significantly increased in the VD+ group, resulting in a richer LAD system.

The less rich network of the vitamin-D-deficient animals was formed by the smaller number of arterioles with diameters of 150–200 μm . On the contrary, the elevated number of larger-diameter (400–550 μm) "small artery" units could not offset this phenomenon.

The extra distance of the individual ring units appeared elevated in the VD+ group. The elevated extra distance has been considered as a hypertensive characteristic so far,

mainly based on studies of retinal hypertensive abnormalities [71,72], but no other hypertensive characteristic (hypertension, network anomalies) was found in the VD+ group, so this may contribute to the elevated ring-unit number and the richer network.

The distribution pattern of the ring elements also shows that tissue perfusion may have improved because the number of small- to medium-diameter vessels distant from the orifice was significantly increased in the VD+ group.

The wall thickness was higher in the VD+ group for medium (100–300) diameters, while the elevation of the wall thickness in the VD− group for the smallest (<50 μm) and largest diameters (400–550) could be seen.

The maximum of the diameter frequency histogram of the VD+ group was shifted upward to 250 from 200 μm, which can also be interpreted as a hemodynamically advantageous alteration.

In our earlier studies [29,31] 200-micrometer-diameter rat coronary-resistance arterioles were examined with pressure arteriography. In vitamin-D-deficient rats, a reduced spontaneous tone was found, and in the fully relaxed state the segments had a narrowed lumen and increased wall thickness. In the present work, segmental geometry was studied along the whole network in pressure-perfused preparations where spontaneous tone could develop. The larger wall thickness we measured in the 200–300 μm range in the substituted animals could be the result of this larger myogenic tone.

The limitation of our study is in the case numbers and the animal study; however, it is also a strength that the genetically homogenous background of the animals resulted in highly homologous networks and a definitive causality of the results. Because of these reasons, only the altered vitamin-D levels could have led to the differences in the network structure. We must mention that both the difficulties of the micropreparation technique and the complexity of the statistical analysis of the vessel rings clearly limited the number of individuals that could be tested. More observers could study a larger number of animals; however, it might lead to greater interobserver variability in the evaluation of network anomalies, which should also be avoided.

5. Conclusions

The wall-thickness elevation in larger branches, the diminishment of the number of smaller units, and the alterations in the bifurcation angles definitively indicate the involvement of vitamin-D receptors in the morphological formation of coronary-resistance-artery networks. Thus, vitamin-D supplementation improves tissue perfusion due to the richer network. In the richer network, the branching pattern was optimal and abnormalities did not increase. While these can be expected to have only moderate hemodynamic effects, pathological processes can be altered by them.

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Article

Vitamin D Deficiency and Gender Alter Vasoconstrictor and Vasodilator Reactivity in Rat Carotid Artery

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Abstract: The vitamin-D-sensitivity of the cardiovascular system may show gender differences. The prevalence of vitamin D (VD) deficiency (VDD) is high, and it alters cardiovascular function and increases the risk of stroke. Our aim was to investigate the vascular reactivity and histological changes of isolated carotid artery of female and male rats in response to different VD supplies. A total of 48 male and female Wistar rats were divided into four groups: female VD supplemented, female VDD, male VD supplemented, male VDD. The vascular function of isolated carotid artery segments was examined by wire myography. Both vitamin D deficiency and male gender resulted in increased phenylephrine-induced contraction. Acetylcholine-induced relaxation decreased in male rats independently from VD status. Inhibition of prostanoid signaling by indomethacin reduced contraction in females, but increased relaxation ability in male rats. Functional changes were accompanied by VDD and gender-specific histological alterations. Elastic fiber density was significantly decreased by VDD in female rats, but not in males. Smooth muscle actin and endothelial nitric oxide synthase levels were significantly lowered, but the thromboxane receptor was elevated in VDD males. Decreased nitrative stress was detected in both male groups independently from VD supply. The observed interactions between vitamin D deficiency and sex may play a role in the gender difference of cardiovascular risk.

Keywords: vitamin D; vitamin D deficiency; cardiovascular disease; carotid artery; vascular reactivity; prostanoid pathway; gender; rat model

1. Introduction

Currently, the role of vitamin D in cardio- and cerebrovascular health is not clear, although disturbances of vitamin D homeostasis—mainly lower vitamin D levels (vitamin D insufficiency and deficiency)—play a role in atherogenesis—atherosclerosis, hypertension

development and different arteriopathies (peripheral arterial diseases, aneurismal arterial diseases) [1]. The prevalence of vitamin D deficiency (25-hydroxy-vitamin D / 25(OH)D < 20 ng/mL or < 50 nmol/L [2]) is high [3] and is expected to grow worldwide; thus, adequate sun-exposure and/or medical vitamin D supplementation are increasingly important. However, a general population-based study, which investigated the intake of fat- and water-soluble vitamins, found that vitamin D intake is the least adequate in both sexes in all age groups [4].

Vitamin D deficiency is a potential risk factor for several cardio-cerebrovascular diseases and events, including stroke [5]. Impaired vitamin D signaling in functionally inactive vitamin D receptor mutant male mice caused compromised cerebrovascular adaptation to unilateral carotid artery occlusion [6]. Four-week-long vitamin D deficient and vitamin D toxic (25(OH)D > 200 ng/mL or > 500 nmol/L [2]) diets resulted in significantly decreased carotid artery diameter and significantly enhanced wall thickness in male rats [7]. Patients with ischemic stroke had significantly lower 25(OH)D levels than control ones and, according to multiple logistic regression, vitamin D considered as a significant predictor in stroke patients and vitamin D deficiency is associated with ischemic stroke [8]. The National Health and Nutrition Examination Survey data evaluation also showed that vitamin D deficiency may be associated with increased stroke risk while higher 25(OH)D levels are associated with reduced stroke risk and these associations were pronounced in the age group of 20–50-year-old women. Moreover, people who have previously had stroke had significantly lower 25(OH)D levels than controls [9]. Among elderly people, a significant linkage was shown between serum 25(OH)D levels and carotid artery distensibility and intima-media thickness (IMT), with these results referring to the possible effects of vitamin D on the functional and structural properties of carotid artery [10]. Suboptimal vitamin D levels (25(OH)D < 30 ng/mL or < 75 nmol/L [2]) and vitamin D deficiency are associated with carotid plaque thickness and with the presence and volume of carotid intraplaque hemorrhage, a parameter which may be a better predictor of unstable plaque and a better estimate of recurrent stroke risk [11].

Stroke is one of the main life-threatening cardio-cerebrovascular and neurological illnesses and its risk also differs between males and females. A retrospective cross-sectional study among stroke patients found that the incidence of stroke and prevalence of stroke risk factors (hypertension, heart diseases, diabetes and hyperlipidaemia) are higher in males than in females [12].

The cerebrovascular consequences of vitamin D deficiency may also be influenced by gender. In healthy men, but not in women, higher total vitamin D intake was associated with decreased cardiovascular disease (CVD) risk [13]. According to the MONICA study, middle-aged women with low (<51.45 nmol/L) 25(OH)D levels had increased risk for stroke and higher total CVD and all-cause mortality during a 17-year-follow-up [14]. Type 2 diabetic patients with carotid atherosclerotic plaque had significantly lower 25(OH)D levels than the control group and 25(OH)D concentrations were inversely correlated with carotid intima-media thickness (CIMT) in men, but not in women [15].

Previous results from our research group also suggest that gender-specific alterations of vascular function and structure can be observed in the renal and cerebral arteries of vitamin D deficient rats [16,17]. In the anterior cerebral artery, vitamin D deficiency resulted in increased wall thickness and testosterone-induced contraction only in male rats [16]. In renal arteries vitamin D deficiency led to impaired acetylcholine (ACh)-induced relaxation in both genders, whereas increased phenylephrine contraction was only found in male animals. Vascular function measurements in the presence of cyclooxygenase (COX) inhibitor and the immunohistochemical labeling of endothelial nitric oxide synthase (eNOS) in these vessels suggest that eNOS and prostanoid pathways may play a role in the gender-specific vascular dysfunction in vitamin D deficiency [17].

Carotid arterial function plays an important role in the regulation of cerebral blood flow and systemic blood pressure by influencing the sensitivity of the high pressure baroreceptor reflex. Monitoring the *in vivo* condition and characteristics of carotid artery,

such as CIMT and carotid plaque area (PA) measurements, are important tools for cardio- and cerebrovascular risk assessment [18].

In the present study, our aim was to examine the possible gender-specific effect of vitamin D deficiency on carotid arteries of rats. The potential role of elastic and contractile elements, eNOS and COX enzymes and a nitrate stress marker were also investigated.

2. Results

2.1. Vascular Function of Carotid Arteries

2.1.1. Phenylephrine-Induced Contraction of Carotid Arteries

Gender-specific difference was observed in the phenylephrine-induced contraction at 10^{-6} mol/L phenylephrine concentration and male gender was associated with more pronounced contraction independently from vitamin D status. Generally, vitamin D deficiency resulted in increased level of phenylephrine-induced contraction (Figure 1A). At 10^{-7} mol/L phenylephrine concentration, male vitamin D deficient vessels showed increased reaction, compared to their female counterparts. Furthermore, vitamin D deficient female arteries were more reactive than female vitamin D supplemented ones. At 10^{-6} mol/L phenylephrine concentration, vessels of vitamin D deficient male rats showed stronger contraction than those of the vitamin D supplemented male animals (Figure 1B).

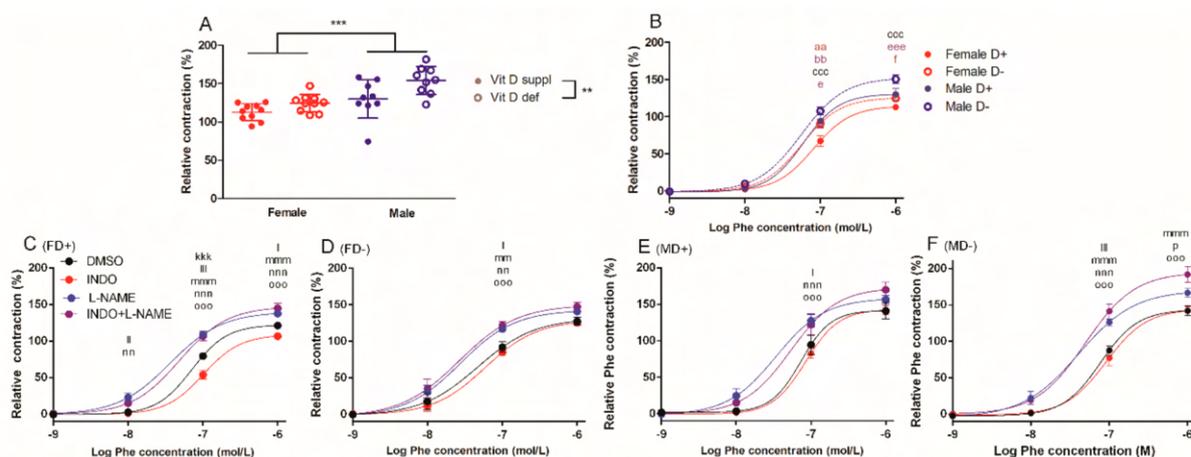


Figure 1. Contraction ability of isolated carotid artery segments: (A) phenylephrine (Phe)-induced contraction in the four experimental groups at 10^{-6} mol/L Phe concentration. Male gender and vitamin D deficiency were associated with more pronounced relative contraction. Data are shown as individual data points; horizontal lines represent mean \pm SD. Two-way ANOVA; factors: gender, vitamin D status. **: $p < 0.01$, ***: $p < 0.001$. (B) Phe-induced contraction. Male rats showed significantly increased contraction compared to females at Phe concentration of 10^{-7} mol/L independently from vitamin D status. Vitamin D deficient female vessels had stronger contraction compared to their vitamin D supplemented counterparts. At Phe concentration of 10^{-6} mol/L, MD– arteries contracted more than FD– ones. Vitamin D deficient male rats showed increased contraction compared to their vitamin D supplemented counterparts. Data are shown as mean \pm SEM; $n = 9$ –11 in each group; repeated measures ANOVA, Bonferroni’s post hoc test; aa: $p < 0.01$ FD+ vs. FD–, bb: $p < 0.01$ FD+ vs. MD+, ccc: $p < 0.001$ FD+ vs. MD–, e: $p < 0.05$ FD– vs. MD–, eee: $p < 0.001$ FD– vs. MD–, f: $p < 0.05$ MD+ vs. MD–. Phe-induced contractions in the presence of L-NAME and/or indomethacin (INDO) or their vehicle DMSO (C) in female vitamin D supplemented rats (FD+) (D) in female vitamin D deficient rats (FD–), (E) in male vitamin D supplemented rats (MD+) and (F) in male vitamin D deficient rats (MD–). L-NAME increased the level of contraction in all experimental groups. Co-incubation with INDO further augmented the contraction only in MD– rats. INDO itself decreased the degree of vascular reaction only in the FD+ group. Data are shown as mean \pm SEM; $n = 5$ –11 in each group; repeated measures ANOVA, Bonferroni’s post hoc test; kkk: $p < 0.001$ DMSO vs. INDO, l: $p < 0.05$ DMSO vs. L-NAME, ll: $p < 0.01$ DMSO vs. L-NAME, ll: $p < 0.001$ DMSO vs. L-NAME, mm: $p < 0.01$ DMSO vs. INDO+L-NAME, mmm: $p < 0.001$ DMSO vs. INDO+L-NAME, nn: $p < 0.01$ INDO vs. L-NAME, nnn: $p < 0.001$ INDO vs. L-NAME, ooo: $p < 0.001$ INDO vs. INDO+L-NAME, p: $p < 0.05$ L-NAME vs. INDO+L-NAME.

In order to explore the role of endothelial nitric oxide synthase (eNOS) and cyclooxygenases in the observed reactivity differences, phenylephrine-induced contraction was repeated in the presence of N(G)-Nitro-L-arginine methyl ester/L-NAME and indomethacin and their combination. L-NAME increased the degree of contraction in all experimental groups. However, indomethacin failed to alter the observed reactivity in the presence of L-NAME in most groups, except the vitamin D deficient males, where indomethacin further increased the contraction compared to L-NAME alone. Indomethacin itself decreased the level of contraction only in the female vitamin D supplemented group, suggesting the significant involvement of constrictor prostanoids in these animals (Figure 1C–F). The lack of indomethacin effect in the vitamin D supplemented male group and in the vitamin D deficient female group may indicate gender and vitamin D dependent regulation of prostanoid effects in vascular contractility.

2.1.2. Acetylcholine-Induced Relaxation of Carotid Arteries

Gender difference was also observed in the acetylcholine-induced relaxation of carotid artery segments at 10^{-6} M acetylcholine concentration (Figure 2A). Vitamin D deficiency failed to alter this function of the vessels in both genders. At this acetylcholine concentration, vitamin D supplemented female vessels had more pronounced relaxation compared to both male groups, while vitamin D deficient females only differed from vitamin D deficient male counterparts (Figure 2B). Nitric oxide inhibition with L-NAME successfully blocked the acetylcholine-induced vasodilation in all experimental groups, while co-incubation with indomethacin had no additional effect. In both male groups, indomethacin itself significantly increased the endothelium-induced relaxation (Figure 2C–F).

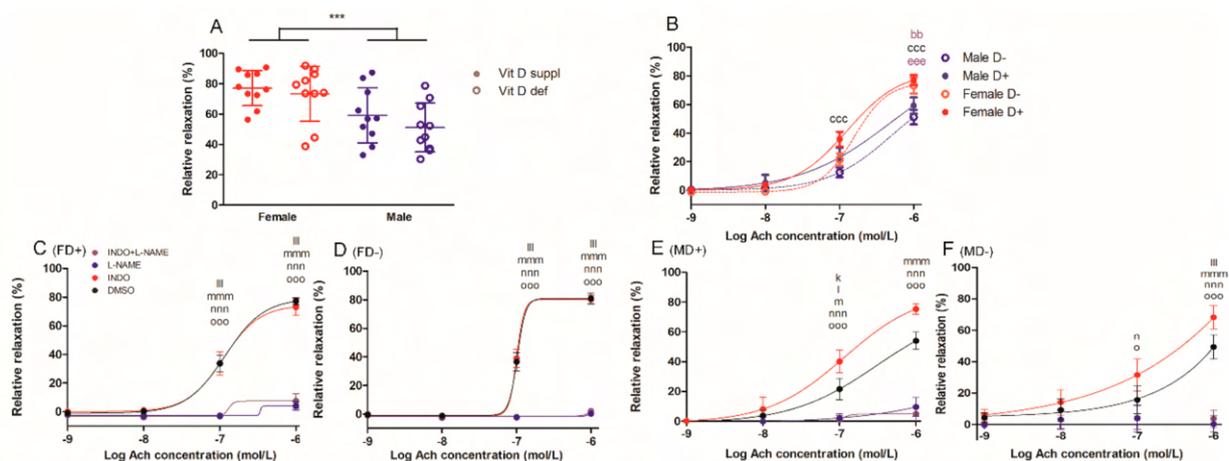


Figure 2. Relaxation ability of isolated carotid artery segments: (A) acetylcholine (Ach)-induced relaxation in the four experimental groups at 10^{-6} mol/L Ach concentration. Male gender was associated with less pronounced relative relaxation. Data are shown as individual data points; horizontal lines represent mean \pm SD. Two-way ANOVA; factors: gender, vitamin D status. ***: $p < 0.001$. (B) Ach-induced relaxation. Male rats showed significantly reduced relaxation compared to females at Ach concentration of 10^{-6} mol/L independently from vitamin D status. Data are shown as mean \pm SEM; $n = 9$ –11 in each group; repeated measures ANOVA, Bonferroni's post hoc test; bb: $p < 0.01$ FD+ vs. MD+, ccc: $p < 0.001$ FD+ vs. MD–, eee: $p < 0.001$ FD– vs. MD–. Ach-induced relaxation in the presence of L-NAME and/or indomethacin (INDO) or their vehicle DMSO (C) in female vitamin D supplemented rats (FD+), (D) in female vitamin D deficient rats (FD–), (E) in male vitamin D supplemented rats (MD+) and (F) in male vitamin D deficient rats (MD–). L-NAME blocked the vasodilation in all experimental groups, co-incubation with INDO had no additional effect. INDO itself increased the degree of relaxation only in both male groups. Data are shown as mean \pm SEM; $n = 6$ –11 in each group; repeated measures ANOVA, Bonferroni's post hoc test; k: $p < 0.05$ DMSO vs. INDO, l: $p < 0.05$ DMSO vs. L-NAME, m: $p < 0.05$ DMSO vs. INDO+L-NAME, n: $p < 0.05$ INDO vs. L-NAME, III: $p < 0.001$ DMSO vs. L-NAME, mmm: $p < 0.001$ DMSO vs. INDO+L-NAME, nnn: $p < 0.001$ INDO vs. L-NAME, o: $p < 0.05$ INDO vs. INDO+L-NAME, ooo: $p < 0.001$ INDO vs. INDO+L-NAME.

2.2. Histological Changes of the Carotid Arteries

The density of elastic fibers in the isolated carotid arteries was significantly decreased by vitamin D deficiency in female rats, but not in males. The marked, but not significantly lower, elastic fiber density of male carotid arteries may have played a role in this phenomenon. On the other hand, the staining intensity observed after the immunolabeling of SMA was decreased by vitamin D deficiency in male, but not in female, carotid arteries. Vitamin D deficiency induced an increase in the immune positivity of thromboxane A2 receptor only in male animals. The optical density of eNOS immunostaining was altered by neither gender nor vitamin D deficiency. Nitratative stress, represented by tyrosine nitration, was slightly lower in both male groups compared to vitamin D deficient female samples (Figure 3A–J).

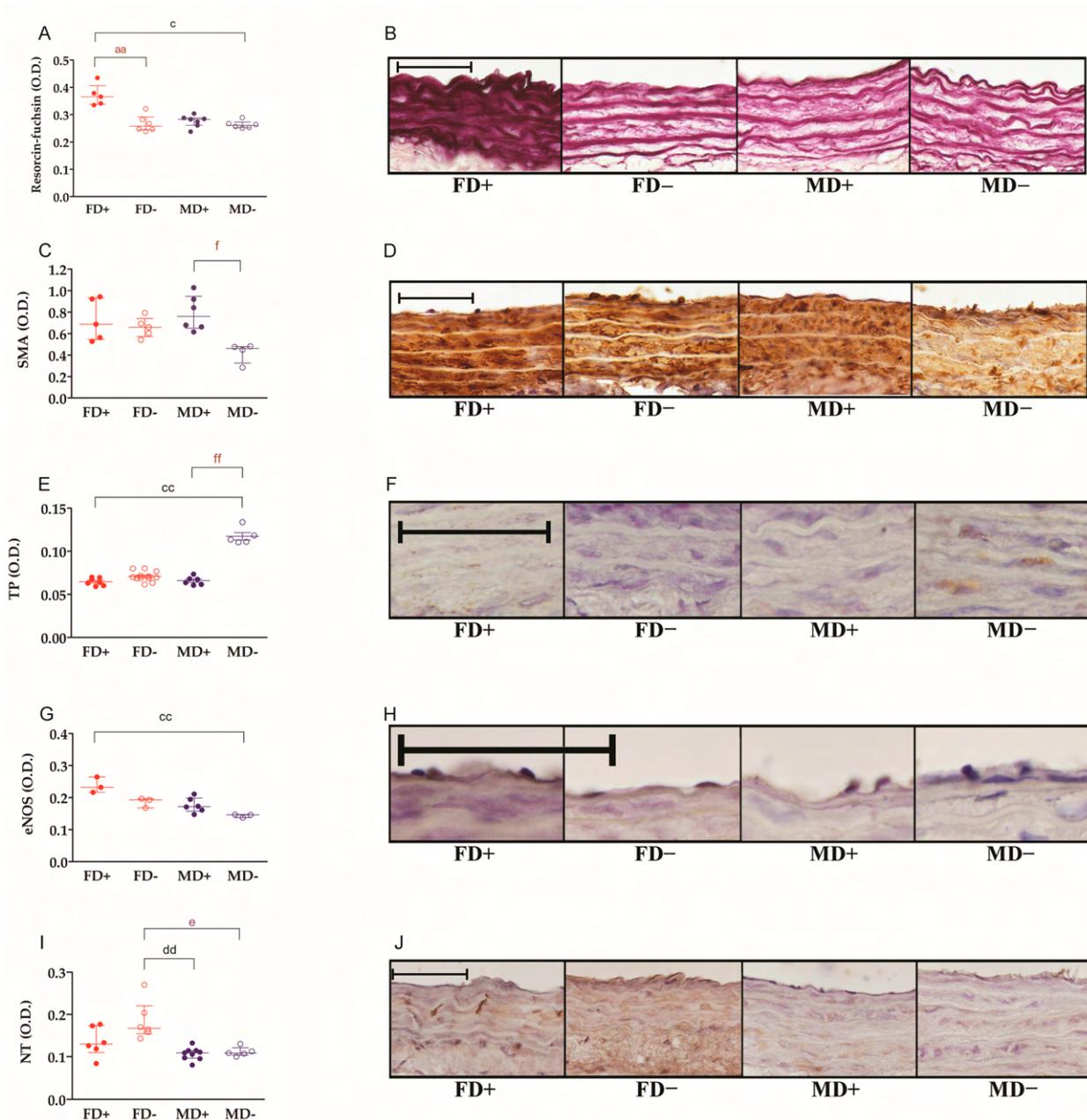


Figure 3. Histological changes of the carotid arteries: (A) elastic fiber density of carotid artery segments. Tissue sections were stained by the purple-colored resorcin-fuchsin stain. Vitamin D deficient female and male arteries showed significantly

lower optical density than vitamin D supplemented female vessels. (B) Representative images of resorcin-fuchsin-stained carotid artery sections. (C) Alpha smooth muscle actin (SMA) immunohistochemical labeling intensity in the media layer of carotid arteries. In vitamin D deficient male rats, the measured optical density was significantly lower compared to vitamin D supplemented male animals. (D) Representative images of carotid arteries stained against SMA. (E) Thromboxane A2 receptor (TP) density of carotid arteries. Vitamin D deficient male rats showed higher receptor density compared to vitamin-supplemented male and female animals. (F) Representative images of TP-stained vessels. (G) Optical density of eNOS labeling in the intimal layer of carotid arteries. Vitamin D deficiency induced a significant decrease in eNOS staining intensity in males. (H) Representative images of vessels labeled with anti-eNOS antibody. (I) 3-Nitrotyrosine (NT) staining intensity of carotid arteries. Both male groups showed lower positivity than vitamin D supplemented female rats. (J) Representative images of NT-stained carotid artery sections. Specific immunohistochemical labeling was visualized by the brown-colored diamino-benzidine (DAB), while blue-colored hematoxylin served as counterstaining. Scale bars show 50 μm . Data are presented as individual data points and lines represent the median [IQR]; Kruskal–Wallis test with Dunn’s post hoc test; aa: $p < 0.01$ FD+ vs. FD–, c: $p < 0.05$ FD+ vs. MD–, cc: $p < 0.01$ FD+ vs. MD–, dd: $p < 0.01$ FD– vs. MD+, e: $p < 0.05$ FD– vs. MD–, f: $p < 0.05$ MD+ vs. MD–, ff: $p < 0.01$ MD+ vs. MD–.

3. Discussion

The main findings of the present study were the following: (i) the carotid artery, which has a primary role in cerebral blood flow and systemic blood pressure regulation, shows gender differences in its reaction to both vasoconstrictor and vasodilator agents; (ii) vitamin D deficiency causes vascular injury in both sexes; and (iii) gender differences can be observed in the pathomechanism of vascular injury caused by vitamin D deficiency.

Recent studies suggest that the vascular function of the carotid artery is influenced by gender. Significantly higher serotonin-induced vasoconstriction was found in male mice compared to females [19]. In addition, these sex-specific alterations are also influenced by the type of vessel under investigation. The release of the cyclooxygenase (COX)-derived constricting factors in mesenteric arterial rings is more pronounced in male spontaneous hypertensive rats (SHR), than their female counterparts [20]. In male rats the sensitivity to endothelin-1’s vasoconstrictor effect on coronary resistance arteries is significantly higher than in females [21]. In males the myogenic tone and the reaction to thromboxane A2 agonist are also more pronounced on coronary resistance arteries [22]. On the contrary, no gender differences were found in renal arteries for Phe-induced contraction [17], and there were also no gender differences in endothelium-dependent vasoconstriction of the popliteal artery [23]. In our current study, we found significantly higher phenylephrine-induced vasoconstriction on carotid arterial rings of male animals independently from their vitamin D status.

Endothelium-dependent vasodilation is also influenced by gender, corresponding to the higher cardiovascular risk of men. The effect of sex hormones on the expression of enzymes involved in the synthesis of endothelium-derived relaxation factors, like eNOS, COX-1 and 2 and prostacyclin synthase, is believed to play a role in this phenomenon [24]. Acetylcholine-induced relaxation on carotid artery rings was found to be significantly higher in SHR female animals than in males, while, in parallel, indomethacin caused increased relaxation in males, while failing to induce change in young females [25]. In our present study, we found no gender difference in eNOS expression of carotid arteries. Our observation that, while in male rats general COX inhibition increased the acetylcholine-induced relaxation, it failed to influence this function in females, suggests that gender-specific alterations of vasoactive prostanoids’ production or action may have played a role in the reduced endothelium-dependent relaxation of the investigated vessels. On the other hand, in the present study, we found no gender difference in TP-specific staining intensity of carotid arteries in vitamin D supplemented rats.

Overall, the weakest vasodilation occurred in the vitamin D deficient male group that was accompanied by the reduced immunohistochemical labeling of eNOS. The production of oxygen-derived free radicals, especially superoxide, may reduce the bioavailability of nitric oxide through their spontaneous reaction forming the potent oxidant peroxynitrite [26]. A characteristic reaction of nitrogen-derived free radicals, especially peroxynitrite, is the

nitration of protein tyrosine residues (NT). On the other hand, we could not show the role of this phenomenon in the reduced endothelium-dependent relaxation induced by male gender. NT positivity was significantly lower in vitamin D deficient male coronary arteries compared to their female counterparts. The known antioxidant effect of testosterone may have contributed to this observation [27].

The risk and the severity of stroke correlate to vitamin D deficiency [28,29]. The increased arterial stiffness that can be observed in vitamin D deficiency may contribute to the increased cardiovascular risk, which can be markedly reduced by vitamin D supplementation (>2000 IU/day) in both sexes [30]. Vitamin D supplementation can dose-dependently decrease arterial stiffness in overweight, vitamin D deficient male and female African Americans [31]. Vitamin D deficiency also contributes to the atherosclerotic transformation of the carotid artery [32]. In our recent study we found that vitamin D deficiency increased the degree of phenylephrine-induced contraction in both sexes. The weakest vasoconstriction was found in female vitamin D supplemented animals. In vitamin D deficient females, increased vasoconstriction was observed that demolished the observed gender difference among the vitamin D supplemented animals. The strongest vasoconstriction was seen in vitamin D deficient males. Similar observations were described in other vessel types. As the consequence of vitamin D deficiency, the myogenic tone of the mesenteric arteries doubles [33]. In coronary arteries of male animals, vitamin D deficiency decreased the vascular reactivity to thromboxane A2 and sexual steroids (estrogen and testosterone) [34]. On the other hand, in renal arteries, we saw increased Phe-induced contraction only in female, but not in male rats [17,26]. Vitamin D can inhibit TP receptor expression [35] and the increased TP immunolabeling intensity observed in vitamin D deficient males may reflect the lack of this suppressing effect, contributing to their increased Phe-induced contraction. Estrogen was also shown to attenuate TP expression [36] that may play a role in the preserved TP expression of vitamin D deficient female carotid arteries. These observations suggest that the consequences of vitamin D deficiency are influenced by gender and the type of the investigated vessel.

Our recent data show that in the carotid arteries of female animals, the balance of the produced prostanoids shifts to vasoconstrictors, as general COX inhibition leads to decreased Phe-induced contraction, which is demolished by vitamin D deficiency. Male vessels also had vasoconstrictor dominance, as indomethacin caused increased Ach-induced relaxation; however, it was not altered by vitamin D deficiency. Interestingly, when indomethacin was applied together with L-NAME, it augmented the level of Phe-induced contraction in vitamin D deficient males. The already described cross-talk between eNOS and COX systems, which was not observable in vitamin D supplemented rats, may have been augmented in this experimental group. In rats fed with standard diet, no interaction was found between these two signaling pathways in the regulation of cerebrocortical microcirculation [37].

As for vasorelaxation, similarly to vasoconstriction, gender differences were observed that might have been affected by the type of the examined vessel. Flow-mediated dilation (FMD) of the brachial artery is not gender-related, while the FMD of the popliteal artery is significantly higher in physically active adult women [38]. Nitroglycerine-mediated dilation is also lower in men's brachial artery [39]. In our current study, we found gender differences in acetylcholine-induced relaxation on carotid arterial rings: male animals showed less relaxation compared to females regardless of their vitamin D status.

Differences in vasoconstriction and vasodilation responses of coronary arteries in this study may be also explained by the observation that both smooth muscle elements and elastic fibers are altered by gender and vitamin D deficiency. The amount of elastic fibers of resistance coronary arteries is significantly lower in male rats than in females [40]. Previous studies examining the effect of vitamin D deficiency on elastic elements showed that abdominal aortic aneurysms have significantly lower levels of elastin in the intima-media composites of male aneurysm walls than of females [41]. Moreover, serious vitamin D deficiency impairs the elastic quality of aorta [42], while in the cerebral arteries this

kind of change cannot be observed, not even in vitamin D deficiency [43]. So, the effect of vitamin D deficiency on elastic fibers can also depend on the type of the investigated vessel. In the present study, we found decreased elastic fiber density in vitamin D deficient male and female carotid arteries, whereas alpha smooth muscle actin immunolabeling was the weakest in the vitamin D deficient male rats. Vitamin D has been previously shown to increase SMA expression in the aortae of rats on a high fat diet [44], which may have played a role in the changes observed in SMA density.

Limitations and strengths: According to our current knowledge, this is the first study which investigated the ex vivo vascular function, histological characteristics and gender differences of female and male rat carotid artery in response to a vitamin D deficient state. The small sample size hindered out investigation of other possibly affected vascular reactivity pathways. The lack of a naive animal group with only a normal diet was another limitation of our study. Further thorough, basic translational and clinical research is needed to clarify the connection between vitamin D deficiency, functional and histological cerebrovascular pathology and its gender difference.

4. Materials and Methods

4.1. Chemicals

Ex vivo vascular functional measurements on isolated rat carotid arteries were performed in Krebs–Ringer solution (in mmol/L: NaCl 119, KCl 4.7, NaH₂PO₄ 1.2, MgSO₄ 1.17, NaHCO₃ 24, CaCl₂ 2.5, glucose 5.5 and EDTA 0.034). The solution was freshly prepared, stored at 37 °C, and bubbled (gas mixture composed of O₂ 20%, CO₂ 5% and N₂ 75%) to maintain stable pH. Chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA–Budapest, Hungary).

4.2. Animals

The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011) and the EU-conforming Hungarian Law on Animal Care (XXVIII/1998). The institutional Animal Care Commission has confirmed the research protocol (IRB: 8/2014 PEI/001/1548-3/2014, PEI/001/820-2/2015).

A total of 48 adolescent (21–28 day old) male and female Wistar rats, weighing 100–140 g, were delivered to the Animal Facility of Semmelweis University in agreement with Charles River (Charles River Ltd., AnimaLab, Vác, Hungary). Rats of both genders were randomly assigned to two further groups, and as a result, four experimental groups were obtained: a female vitamin D supplemented group (FD+), a female vitamin D deficient group (FD−), a male vitamin D supplemented group (MD+) and a male vitamin D deficient group (MD−); *n* = 11–13 in each group.

4.3. Chronic Treatment of the Rats

In order to induce vitamin D deficiency via reduced intake, rats in the corresponding groups were fed ad libitum with vitamin D Free Lab rat/mouse chow (Ssniff Spezialdiäten GmbH, Soest, Germany) containing less than 5 IU/kg vitamin D for eight weeks resulting in vitamin D deficiency (below 10 ng/mL) [43,45]. Rats in vitamin D supplemented groups were fed ad libitum with a normal chow containing 1000 IU/kg of vitamin D. Furthermore, oral administration of additional vitamin D through a gavage cannula was applied to ensure the targeted plasma vitamin D levels [43,45]: 500 IU cholecalciferol on the second week and a weekly maintenance dose of 140 IU/100g on the fourth, fifth, sixth and seventh weeks (Vigantol (cholecalciferol) 20,000 IU/mL, Merck/Merck Serono, Darmstadt, Germany). The treatment protocol resulted in four experimental groups; vitamin D supplemented female and male (FD+, MD+) and vitamin D deficient female and male (FD−, MD−) groups. The animals had access to tap water ad libitum. Rats were housed at constant room temperature (22 ± 1 °C) with a 12 h/12 h light–dark cycle. All experimental groups had normal blood pressure [45,46]. Serum 25(OH)D levels of the animals were the following:

FD+: 32.328 ± 4.49 ng/mL; FD–: 6.044 ± 0.63 ng/mL; MD+: 19.66 ± 0.81 ng/mL; MD– rats: 3.59 ± 0.21 ng/mL [43,45]. Final body weight, weight gain and serum testosterone levels were not significantly influenced by vitamin D status in either gender [43,45,46].

After 8 weeks, rats were anesthetized with Nembutal (45 mg/kg intraperitoneal (i.p.)), perfused with heparinized nKR solution for 2 min. Carotid arterial segments were cut into five equal rings (2 mm long), four of which were placed on a conventional wire myograph setup, while the fifth vascular ring was fixed in formaldehyde and embedded in paraffin.

4.4. Myography

A conventional wire myograph system was adopted to measure the isometric tension of isolated carotid arterial rings (610-M MultiMyograph System, Danish Myo Technology, Aarhus, Denmark, with Lab-Chart Evaluation System, AD Instruments, Oxford, UK–Ballagi Ltd., Budapest, Hungary). The organ chambers were filled with nKR solution, kept at 37 °C and bubbled (gas mixture composed of O₂ 20%, CO₂ 5% and N₂ 75%) to maintain stable pH. Following the development of stable pre-tension (15 mN), the contractility of the vessels was obtained when applying 124 mmol/L K⁺, which served as the reference value of the contraction forces. Vascular rings were equilibrated in nKR, and accumulative doses of phenylephrine (10^{-9} – 10^{-6} mol/L) were administered to induce contraction. Acetylcholine-induced vasodilation was examined by incubating the vessels with increasing doses of Ach (10^{-9} – 10^{-6} mol/L), subsequent to Phe precontraction (10^{-6} mol/L). Measurement of Phe-induced contraction and Ach-induced vasodilation was repeated in the presence of the nitric oxide synthase inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME) (10^{-4} mol/L) and the general COX inhibitor indomethacin (10^{-5} mol/L) or their vehicle DMSO.

4.5. Immunohistochemistry

Carotid arterial tissue sections were stained with resorcin-fuchsin (RF). Immunohistochemistry was performed to label alpha smooth muscle actin (SMA), thromboxane A2 receptor (TP), endothelial nitric oxide synthase (eNOS) and 3-nitrotyrosin (NT). Antigen retrieval was performed by heating the slides in citrate buffer (pH = 6) following deparaffinization. Then, 3% H₂O₂ in dH₂O was applied to block endogenous peroxidase activity. Non-specific labeling was prevented via utilization of 2.5% normal horse serum (Vector Laboratories, Burlingame, CA, USA). Primary antibodies (SMA: 1:10,000; eNOS: 1:50 (Abcam, ab46545 and ab76198, Cambridge, UK)), TP 1:50 (MyBioSource, MBS2032166, San Diego, CA, USA) and NT 1:500 (Merck Millipore–Sigma-Aldrich, 06-284, Budapest, Hungary) were applied overnight at 4 °C. For SMA and eNOS horseradish-peroxidase-linked anti-mouse was used for secondary labeling, while for TP and NT anti-rabbit polyclonal horse antibody (Vector Laboratories, MP-7402 or MP-7401, Burlingame, CA, USA) was used for secondary labeling. Visualization of specific labeling was accomplished by brown-colored diaminobenzidine (DAB) (Vector Laboratories, SK-4100, Burlingame, CA, USA), while blue-colored hematoxylin served for counterstaining (Vector Laboratories, H-3404-100, Burlingame, CA, USA). Brightfield microscopy images were acquired using a Nikon ECLIPSE NI-U microscope and Nikon DS-Ri2 camera (Nikon Corporation, Minato City, Tokyo, Japan) with 40x objective. Non-calibrated optical density of the media layer of resorcin-fuchsin-stained vessels was obtained for the purpose of assessing the density of non-contractile elements using ImageJ software (National Institutes of Health (NIH), Bethesda, MA, USA). The measurements of the non-calibrated optical density of specific staining in the intimal or medial layers of the vessel wall were also completed with ImageJ software in the case of immunohistochemical labeling.

4.6. Statistical Analysis

Repeated measures two-way ANOVA was completed using Bonferroni's post hoc test with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) for the analysis of vascular function curves. At a certain agonist concentration, vascular reactivity was

analyzed by two-way ANOVA (gender, vitamin D status). The Kruskal–Wallis test with Dunn’s multiple comparison test was applied to accomplish the comparison of histological and immunohistochemical evaluations of the experimental groups. $p < 0.05$ was uniformly accepted as the threshold for statistical significance. Data are presented as the mean \pm SEM or median [IQR].

Significance symbols (Table 1):

Table 1. The number of the symbols (x) indicates the strength of the significance: x: $p < 0.05$, xx: $p < 0.01$; xxx: $p < 0.001$. The meanings of the letter colors are as follows: gender difference is indicated by violet (b,e), while burgundy highlights a significant difference through different vitamin D statuses (a,f).

Between the Groups		Between the Inhibitors	
a:	FD+ vs. FD−	k:	DMSO vs. INDO
b:	FD+ vs. MD+	l:	DMSO vs. L-NAME
c:	FD+ vs. MD−	m:	DMSO vs. INDO+L-NAME
d:	FD− vs. MD+	n:	INDO vs. L-NAME
e:	FD− vs. MD−	o:	INDO vs. INDO+L-NAME
f:	MD+ vs. MD−	p:	L-NAME vs. INDO+L-NAME

5. Conclusions

Differences in cerebral blood flow, as well as gender and vitamin D dependent differences of stroke risk, can be partially explained by the local changes of circulatory control. In the present study, vitamin D deficiency resulted in vascular impairments in both genders, although gender differences in the pathomechanism could be observed. Both vitamin D deficiency and male gender resulted in increased contraction of carotid artery. Decreased relaxation reactivity of the carotid arteries was observed in male gender independently from their vitamin D status. Inhibition of prostanoid signaling reduced contraction in females, but increased relaxation ability in male rats. These functional changes were accompanied by vitamin D and gender-specific structural and protein expression alterations, which were also characteristic of the examined vessel type, the carotid artery.

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