DOI:10.14753/SE.2022.2648

EFFECTS OF GENDER AND VITAMIN D DEFICIENCY ON RENAL AND CAROTID ARTERIES

PhD thesis

Miklós Sipos MD

Doctoral School of Clinical Medicine
Semmelweis University

Supervisor: Szabolcs Várbíró, MD, DSc
Consultant: Eszter Mária Horváth, MD, PhD

Official reviewers: Endre Zima, MD, DSc
István Sziller, MD, PhD

Head of the Complex Examination Committee: Péter Várnai, MD, DSc
Members of the Complex Examination Committee: Zsolt Kopa, MD, PhD

Gábor Vermes MD, PhD

Budapest
2021

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LIST OF ABBREVIATIONS

20-HETE	20-hydroxyeicosatetraenoic acid
Ach	Acetylcholine
AII	Angiotensin II
ANOVA	Analysis of Variance
AT1	Angiotensin II receptor type 1
COX	cyclooxygenase
CVD	cardiovascular disease
DAB	diamino-benzidine
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
EDCF	endothelium-derived contracting factors
eNOS	endothelial nitric oxide synthase
FD-	Vitamin D deficient female
FD+	Vitamin D supplemented female
FMD	flow mediated dilatation
IMT	intima-media thickness
INDO	indomethacin
L-NAME	N(G)-Nitro-L-arginine methyl ester
MD-	Vitamin D deficient male
MD+	Vitamin D supplemented male
NO	nitrogen monoxide
NT	3-nitrotyrosine
PGI2	prostaglandin I2
Phe	Phenylephrine
RAAS	renin-angiotensin-aldosterone system
RF	resorcin-fuchsin
SHR	spontaneous hypertensive rat
SMA	smooth muscle actin

TP	thromboxane A2 receptor
VD	Vitamin D
VDD	Vitamin D deficiency
VDR	Vitamin D receptor

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1. INTRODUCTION

1.1. Gender differences in cardiovascular risk

Results from both human trials and animal studies have established that there is a significant gender difference between males and females in blood pressure control and in the susceptibility, prevalence, pathophysiology, clinical presentation, response to treatment and outcome of cardiovascular diseases (CVDs) (1, 2). Pre-menopausal women tend to have a generally more favorable metabolic, cardiovascular and sympathetic (lower sympathetic activity) profile compared to age-matched men, and they are also more resistant to obesity-related hypertension and cardiometabolic diseases (3). Meanwhile, the decrease in oestrogen levels (menopause) in middle-aged and elderly women leads to fat storage in the abdominal region and an increase in sympathetic activity, which raises the prevalence of hypertension and the risk of cardiometabolic disease, that is higher in elderly women than in elderly men (3).

Gender differences occur not only in the prevalence, severity or outcome of disease between men and women, but there are also significant sex differences in the morphology and function of blood vessels, which may explain the differences between men and pre-menopausal women. Sex hormones, such as estrogens and androgens affect vascular function, the expression and activation of various vascular receptors, and even sexual steroids themselves cause acute vasorelaxation in blood vessels (4).

1.1.1. Sex hormone related differences in vascular reactivity

Due to their location and size, blood vessels fulfill a different function in the vascular system; the larger conductive arteries are primarily responsible for transmitting blood to the organs, ensuring a balanced blood supply; whereas the smaller resistance vessels are responsible for regulating the distribution of blood to meet regional needs. Therefore, when examining the contraction characteristics of arteries and their gender differences, we should always take into consideration the type and size range of the studied vessel.

Gender differences in vasoconstriction have been already documented related to most vessels; gender difference can be seen in case of mesenteric and coronary arteries. The release of cyclooxygenase-derived contracting agents on mesenteric arterial rings is

stronger in male than in female SHR animals (5). In resistance coronary arteries, male animals have increased myogenic tone, stronger response to thromboxane agonist and enhanced sensitivity to endothelin-1 compared to females (6, 7). However, no sex differences were found in endothelium-dependent vasoconstriction of the popliteal artery (8).

Like contraction, in case of blood vessel relaxation, the observed gender difference is also affected by the type and size range of the investigated blood vessel. This is clearly obvious from flow-induced dilation (FMD) studies, as there is no difference in FMD between men and women in brachial arteries, while in popliteal arteries, flow-induced dilation is significantly higher in physically active women compared to men (9).

Not only as a result of exercise, but also in many diseases, we can find gender differences in endothelial function. For example, in chronic kidney disease, young women had greater endothelial dilatation compared to men of the same age, while in elderly, this gender difference was no longer observed, suggesting that the rate of endothelial dilatation decreases more expressly in women during aging (potentially it can be a menopausal effect), than in men with chronic kidney disease (10). While in hypertriglyceridemia, women have higher FMDs than men (11). Furthermore, we also see a gender difference in FMD in healthy children and adolescents: FMD is lower in boys than in girls (12).

Vessels of major importance for cardiovascular disease include the carotid artery, a key vessel in the cerebral circulation, which is involved in the pathomechanism of stroke, and the renal artery, a vessel supplying the renal circulation, which may play a role in the pathomechanism of hypertension. Sex differences in the function of these blood vessels have been partially investigated previously: in carotid arteries, serotonin-induced contraction is higher in male animals, but no sex difference was found in the response to thromboxane agonist (13). No difference appeared in vasopressin-induced contraction of renal arteries between male and female rats (14). No differences in acetylcholine (Ach)-induced relaxation in carotid arteries have previously been found between male and female littermate control (eNOS(+/+)) and heterozygous (eNOS(+/-)) mice (10), while no sex differences in Ach-induced relaxation in renal arteries have previously been found either (15).

1.2. Vascular impacts of vitamin D deficiency and supplementation

The prevalence of vitamin D (VD) deficiency (VDD) is very high and is expected to increase worldwide, pointing to adequate VT supplementation increasingly important (16). Literature suggests that VD supplementation in men and women is inadequate regardless of age (17).

The body's vitamin D supply can be measured by serum $25(OH)D_3$ levels, which are also influenced by lifestyle, seasons, amount of sunshine, genetic factors, and skin pigmentation (18). There is no complete agreement on the lower limit of normal serum VD level, but 30 ng / ml (75 nmol / l) $25(OH)D_3$ is considered appropriate based on the guideline of the Endocrine Society and the latest Hungarian consensus meeting. (18, 19). Deficiency is below 30 ng/ml, deficiency symptoms occur below 20 ng/ml. To maintain serum levels of 30–32 ng/ml, we need 2200–3000 IU of vitamin D per day from an external source (ultraviolet radiation, food, dietary supplements) (20). The conversion method between the nmol/l and ng/ml is the following: SI: nmol / l = 2.5 * ng / ml.

VDD is a potential risk factor for many cardio-cerebrovascular diseases, including hypertension and stroke (21, 22). However, randomized controlled clinical trials have so far failed to demonstrate direct beneficial effects of VD on cardiovascular events (23). In one of the most recent studies (VITAL), 2000 IU of VD per day in combination with 1 g of omega-3 fatty acid per day had no effect on major and minor cardiovascular events after 5 years of follow-up, only a protective effect of omega-3 supplementation was demonstrated (24). However, it should be noted that in this study the placebo group was not VD deficient, as their average plasma 25-OH-D3 level was around 30 ng/mL (24). The discrepancy between these observations may be the results of the different reactivity of patient subgroups.

VD directly affects the function and morphology of blood vessels. The adverse effects of vitamin D deficiency also depend on how severe the vitamin D deficiency was and for how long did it take. A structurally rebuilt vessel wall (e.g. vessel wall thickening) may not be able to fully recover as a result of subsequent vitamin D supplementation, however vitamin D supplementation may nevertheless can have beneficial effect: 4 months of vitamin D treatment (> 2000 IU / day) reduced arterial vessel wall stiffness in the initially vitamin D-deficient group (25).

In elderly humans, a significant correlation between VDD and carotid artery dysfunction and intima-media thickness is observed (26). Pharmacological reactivity of coronary resistance arteries in male rats is impaired by VDD: thromboxane A2-induced vasoconstriction and testosterone- and 17-beta-oestradiol-induced relaxation are significantly reduced, with a parallel reduction in thromboxane receptor (TP) expression in the vessel wall (4). Deficiency of VD in the cerebral arteries of female animals reduces myogenic tone (27).

The beneficial effects of VD supplementation on vascular relaxation have been previously observed both by in vitro and in vivo studies (28, 29). In a hypertensive patient group where renal arteries were studied, impaired endothelium-dependent relaxation was partially restored by in vitro calcitriol incubation 12 hours, with improvement in relaxation accompanied by normalization of oxidative stress-related proteins such as NOX-2, NOX-4, p67phox and SOD-1 and reduced levels of reactive oxygen derivatives (28). Furthermore, eNOS expression increases dose-dependently on ergocalciferol treatment (29). Calcitriol prevented the up-regulation of NOX-2, NOX-4, p67phox proteins and the increase of reactive oxygen derivatives in normotensive patients and in human aortic endothelial cell cultures (28). These effects have been observed not only in human studies, but also in animal models such as spontaneous hypertensive rats and Wistar rats with AII-induced hypertension (28).

Vasodilation, as a vascular effect of $1,25(OH)_2D_3$ caused by the decrease of nitric oxide (NO)-bounding oxygen radicals production and increase of NO bioavailability (30, 31).

1.3. Gender differences in vascular effects of Vitamin D

VD sensitivity shows gender differences in cardiovascular system and some local vascular regions (4, 27, 32-36). The higher VD intake is associated by reduced cardiovascular risk in healthy men, while Sun et al. found no such effect in women (37). Although MONICA study found that middle aged women with low VD levels (<20,58 ng/ml) had an increased risk for stroke, total CVD and all-cause mortality during a 17-year-follow-up (38). According to a retrospective cross-sectional study the incidence of stroke and risk factors for stroke - such as high blood pressure, heart disease, diabetes and hyperlipidaemia - are higher in men than in women (39). Furthermore, the stroke knowledge is greater among men, but the prestroke health behavior is worse, than among women (40). Patients with ischaemic stroke have lower levels of 25(OH)D VD than their counter group and a multiple logistic regression suggests that low VD level correlates with an increased risk of stroke (41). The literature indicates that VD has an effect on cardiovascular disease, and a significant proportion of these also shows gender-related difference.

1.4. Animal models of Vitamin D deficiency

One of the disadvantages of human studies is that it is very complicated and difficult to select experimental groups where it is exactly known how long and how severe VDD has been present, which impacts the assessment of the lesions seen. Laboratory animals, animal models of VDD effectively eliminate these problems, and the changes observed can only be attributed to the effect of VDD and supplementation. The animal model provides the possibility to adjust precisely the amount of VD supplementation, without the unpredictable effect of consumption VD-containing foods or sun exposure, that can affect the results in human. Animal models of VDD can be divided into 2 groups: genetically modified vitamin D deficient animal models and animals fed with a Vitamin D-free diet. The basic idea of the last animal model is to feed the rodents exclusively on a vitamin D-free diet for several weeks. There are several types of Vitamin D-free foods that have been proven to contain no VD (< 5 IU/kg or 0 IU/kg) (42). Having been treated for eight weeks, the animals have lower serum $25OHD_3$ level than 6 ng/mL (33, 35). The advantage of this method is that it is cheap and more similar to VDD in human studies -

low VD levels in humans are not the result of genetic reasons, but mainly of lifestyle and nutritional factors.

In genetic models, either Vitamin D receptor (VDR) or the gene of an enzyme (e.g. alpha-hydroxylase) that plays a key role in its metabolism is modified or knocked out (43, 44). An animal model of Vitamin D-dependent rickets type II, (VDDR II) in which animals are normal phenotypic at birth but develop hypocalcaemia, hyperparathyroidism and alopecia in the first month of their life (45). The advantages of genetic models are that we can selectively investigate the effects of VDD in different organs and the effects of VDD on embryogenesis. The disadvantage of genetically modified animals is their very high cost.

1.5. The long term effects of steroid hormones on the cardiovascular system

Steroid hormones, such as sex hormones (androgenic and estrogenic hormones), or vitamin D have not just acute or so-called non-genomic effects (e.g., acute effects on eNOS function) but also have a long-term, chronic effect on the body. This is called a genomic effect, that develops slowly, hormones, by binding to their own receptors (estrogen receptors, androgen receptors, VDR) modulate gene transcription and protein synthesis as transcription factors (46, 47).

The genomic effects of testosterone include increasing myocardial volume and cardiac output, possibly by reducing left ventricular afterload (48, 49). Furthermore, androgens enhance the proliferation of smooth muscle cells, which are also found in the walls of blood vessels (50). Furthermore testosterone also increases the synthesis of angiotensin-converting enzyme (51).

Estrogens have a complex effect on myocardium and cardiac function. Estradiol 17-beta-oestradiol (E2) and its receptors estrogen receptor alpha (ERα) and beta (ERβ) are involved in the development of physiological and pathological myocardial hypertrophy. In case of pathological cardiac enlargement, the myocardial hypertrophy is inhibited by a selective ERα agonist in female ovariectomized mice, whereas deletion of ERβ enhances the rate of myocardial hypertrophy in both sexes (52, 53). In physiological myocardial hypertrophy, deletion of ERβ inhibits the development of exercise-induced hypertrophy in both sexes (54). Furthermore, estrogens inhibit smooth muscle cell

proliferation in the vessel wall (55) and decrease the synthesis of angiotensin-converting enzyme and the expression of angiotensin 1 (AT1) receptor (56).

Left ventricular hypertrophy is a very common complication in chronic kidney disease, the development of which is inhibited in an experimental animal model by vitamin D receptor activators (calcitriol, paricalcitol and alfacalcidol). Cardiomyocyte size, left ventricular wall and septum thickness, and heart/body weight ratio also decrease. (57). In the absence of vitamin D, RAAS activation increases (58), whereas vitamin D decreases AT1 receptor expression and smooth muscle cell proliferation, thus reducing the development of athoresclerosis (28, 47).

Cardiovagal baroreflex sensitivity decreases physiologically with advancing age, which is similar in men and women, however, in pathological conditions such as hypertension or type 2 diabetes, the sensitivity of cardiovagal baroreflex also decreases and the rate is more pronounced in women than men. Sympathetic baroreflex sensitivity decreases in elderly women, with concomitant increases in arterial vessel wall stiffness similarly to men. This decrease in sympathetic baroreflex sensitivity may predispose to the development of hypertension and cardiovascular disease in elderly women (59). In chronic heart failure, long-acting testosterone increases baroreflex sensitivity in elderly patients (60). In early menopause, transdermal estrogen and intermittent micronized progesterone therapy increases baroreflex sensitivity (61). No significant difference was found between $25OHD_3$ and baroreflex sensitivity, suggesting that VD does not alter basal parasympathetic nervous system activity (62).

1.6. Methods of testing vasculature

1.6.1. In vivo measurement methods

Several methods are available for the morphological, biomechanical and functional study of the arteries. There are non-invasive methods (arteriography, US, CT, MRI), that can be used even in clinical trials, and there are invasive methods – mostly used in animal models - which can help to get more accurate picture of the adaptation of the blood vessels. (63). Flow-mediated vasodilation (FMD) can provide information on endothelial function. The brachial artery is examined the most often by this method (64). Parameters characterizing arterial stiffness (augmentation index, pulse wave velocity) can be

measured by arteriography (65), the 24-hour monitor version of which is the Mobil-O-Graph, that has been tested by our group and based on its experience we plan to conduct our clinical trials. The measurement of intima-media thickness of the carotid artery (duplex ultrasound) can be used to evaluate atherosclerosis of the entire vasculature (66). Besides their simplicity, another advantage of these tests is that they are painless and non-invasive.

1.6.2. In vitro measurement methods

The best-known in vitro techniques for investigating different vessels are wire-myography and pressure-myography, which can be used to examine isolated vascular rings or segments, and it provides the possibility of examining even very small vessels (external diameter < 200 μm). The wire myograph measures the isometric contraction of blood vessels, i.e. the level of tension at a constant fiber length, which can be increased or decreased by different vasoactive substances. The vasoconstrictor will increase the tension, yet the vasodilator will decrease it (67-70). At the beginning of the experiment, the maximum tension can be determined using K+ solution, with which the effects of the different pharmacological or inhibitory agents can be compared later (36, 71).

With a pressure myograph, we can examine isolated vascular segments to determine not only the function of blood vessels, but also their morphology and biomechanical function. In brief, it works by placing the prepared, lateral branch-free vascular segment in an organ bath, cannulating both ends of the arteriolar segment, and connecting the microcannulas to a servo-controlled pump to provide constant pressure and to alter the intraluminal pressure during the experiment. Constant temperature, oxygenation and pH are maintained in the organ bath. According to the different experimental protocols, it is possible to either inject the vasoactive pharmacones into the organ bath or to test the pharmacones intraluminally. The glass-bottom organ bath is placed on the stage of an inverted microscope and images of the vessel segment are taken with a high-resolution digital camera, which can be analyzed off-line using image analysis software: measuring the outer and inner diameter of the vessels and the wall thickness (7, 27, 72).

In vitro investigations include also histological and immunohistochemical examination of blood vessels, which can be used to identify the composition of the vessel wall and the number of different receptors. For classical histological studies, the dissected vessels are

placed in formalin and after dehydration, the histological samples are embedded in paraffin and the resulting blocks can be sectioned at 4-5 μm using a microtome. The sections can be labelled with different stains, such as resorcinol-fuchsin staining for fiber composition or picrosirius staining for collagen (73). In immunohistochemical studies, the paraffin-embedded sections are also stained with specific antibodies after deparaffinisation and antigen detection. By using specific antibodies, it is possible to target different proteins, polysaccharides, lipids or glycoproteins in cells or in the extracellular space, which can be receptors, hormones, enzymes, white blood cell types, lectins, etc. Antibody detection can be direct or indirect: in direct detection the antibody is labelled, in indirect detection a secondary antibody produced against the antibody is bound and detected by a special technique such as the avidin-biotin-enzyme complex technique (74-76).

DOI:10.14753/SE.2022.2648

2. OBJECTIVES

VDD is a significant public health problem, low VD level is associated with hypertension and adverse cardiovascular events. Gender difference in cardiovascular risk between men and women has been known for a long time.

The relative protection of women before menopause is clearly due to the effect of estrogen. In women, due to a decrease in estrogen levels after menopause, the cardiovascular risk increases, which may exceed that of men of the same age. Testosterone has two facial effects: it increases cardiovascular risk in men even at too low and too high levels.

Furthermore, also sex difference can be found in the effect of VD supplementation and deficiency on cardiovascular events.

Our team therefore intended to investigate in detail the functional and morphological adaptations of renal and carotid blood vessels in VDD and VD supplementation and the gender differences in an animal model. VDD was reached by feeding the rats with VD free chow for 8 weeks (Vitamin D Free Lab rat/Mouse Chow (ssniff Spezialdiaten GmbH, Soest, Germany) containing less than 5 IU/kg VD). In case of Vitamine D supplemented animals fedded with conventional chow, containing 1000 IU/kg of VD oral administration of additional VD through a gavage cannula was applied to ensure the targeted plasma VD levels (25-50 ng/ml); 500 IU cholecalciferol on the second week and a weekly dose of 140 IU/100g on the fifth, sixth and seventh weeks (Vigantol (cholecalciferol) 20000 IU/ml, Merck/Merck Serono, Darmstadt, Germany).

Having treated the animals for 8 weeks, we assessed the function of renal and carotid vessels by a conventional wire myograph, when we measured the isometric tension of isolated renal and carotid arterial rings. Phe-induced contraction and Ach-induced relaxation was measured in isolation, and was repeated in the presence of a generalized COX inhibitor (indomethacin), a specific COX-2 inhibitor (NS398) and nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor. Finally our measurements were completed with histological analyses in order to identify the pathomechanisms behind the observed deviations.

This raises the question whether there are gender differences in the effects of VDD and supplementation on the function of the two key blood vessels, the renal and carotid arteries in the terms of cardiovascular diseases.

To find answers for the following questions we used a Whistar-rat model of VD deficient and VD supplemented male and female animals:

1) Are there any sex related differences in the function of renal and carotid artery of Vitamin D supplemented young adult male and female rats?
2) Are these characteristics changing by Vitamin D deficiency?
3) Are there gender differences in VDD induced changes?

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3. RESULTS

3.1. Renal arteries

3.1.1. Vascular Function of Renal Arteries

3.1.1.1. Gender differences in contraction

Vascular function, contractile force and relaxation ability of renal arteries were examined by wire-myograph. Phenilephrine-induced (Phe) contraction was retained in all groups. No gender differences were found in Phe-induced contraction of the renal arteries of VD supplemented male and female animals **(Figure 1)**. The COX 2 inhibitor (NS398) and the general COX inhibitor (INDO) significantly reduced the contraction in arteries of vitamin D-supplemented female and male rats, which was more significant for COX-2 in both sexes, no sex difference was observed in this respect **(Figure 2A and 3A)** The sex had no influence on Angiotensin II-induced (AII) contraction **(Figure 4)**.

3.1.1.2. The effect of VDD on contraction

As a result of VDD female rats showed significantly increased Phe-induced contraction at concentration of 10^{-7} mol/L, while Phe-induced contraction of males was not affected by VDD **(Figure 1).** Indomethacin and NS398 pre-treatment significantly reduced the level of contraction in VD deficient male and female groups. In males the inhibitory effect of COX-2 was significantly higher compared to indomethacin **(Figure 3A and 3B)**.

3.1.1.3. Gender differences in the effect of Vitamin D deficiency

Phe-induced contraction was significantly higher in renal arteries of VD deficient females compared to both VD deficient and VD supplemented males at concentrations of 10^{-7} mol/L **(Figure 1)**. Although in both sexes COX-2 inhibitor and general COX inhibitor pre-treatment significantly reduced the level of contraction, it should be emphasized that in males COX-2 inhibition had a significantly higher inhibitory effect compared to indomethacin, while in females such effect was not observed **(Figure 2B and 3B).** Angiotensin II-induced contraction was significantly decreased both in VD deficient male and female groups at concentrations of 10^{-7} mol/L compared to VD supplemented males **(Figure 4)**. However AII induced contraction of VD deficient females was also significantly lower than it was in VD supplemented males **(Figure 4**).

DOI:10.14753/SE.2022.2648

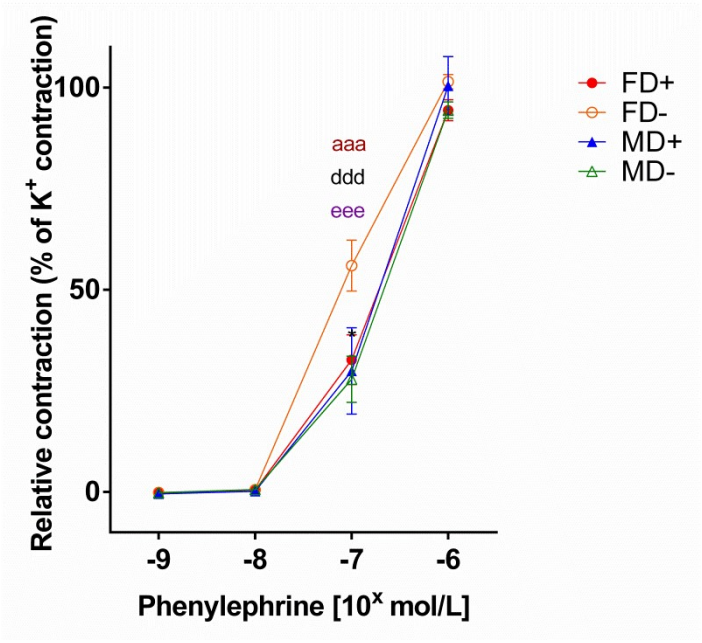

Figure 1. Phenylephrine (Phe) induced contraction

VD deficient female rats showed significantly increased contraction at Phe concentration of 10-7 mol/L compared to their VD supplemented female counterparts. Furthermore, VD deficient female rats showed significantly increased contraction compared to male groups – independently of VD status. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD−), VD supplemented male group in blue (MD+) and VD deficient male group in green (MD−). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status. Data are shown as mean ± SEM; N = 8 in each group; **aaa**: $p < 0.001$ FD+ vs. FD−, ddd: $p < 0.001$ FD−vs. MD+, **eee**: $p < 0.001$ FD− vs. MD−. (Sipos et al. 2021a77)

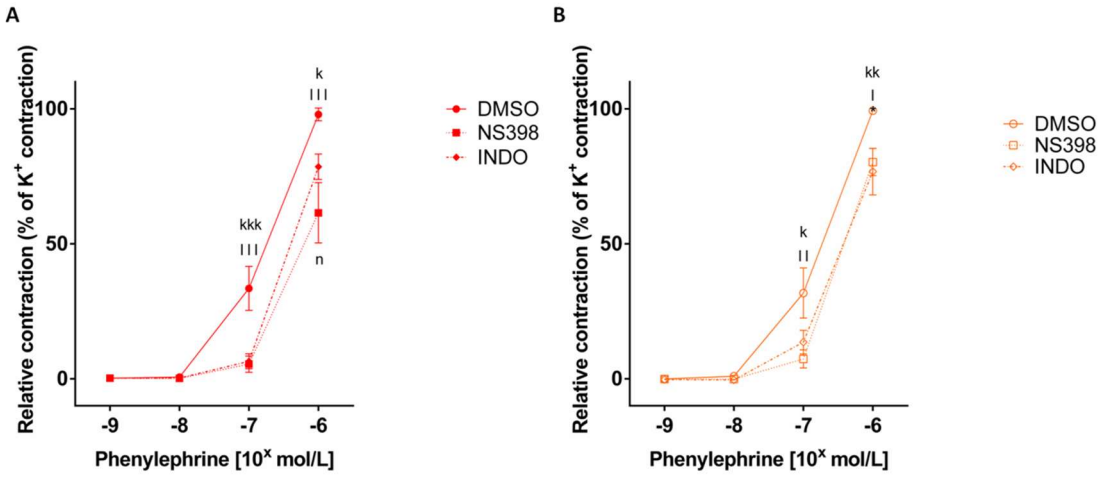

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Figure 2. Phenylephrine (Phe) induced contraction in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in female Vitamin D supplemented and female Vitamin D deficient rats

A) Female Vitamin D supplemented group. In case of Vitamin supplementation, both general COX, and specific COX-2 inhibition led to decreased contraction that was more pronounced in case of specific COX-2 inhibition. **B) Female Vitamin deficient group.** However, in case of VDD, both indomethacin and NS398 pretreatment resulted in reduced contraction force. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD–). Data are shown as mean ± SEM; N = 8 in each group; kkk: $p < 0.001$, kk: $p < 0.01$, k: $p < 0.05$ INDO vs. DMSO; lll: $p < 0.001$, ll: $p < 0.01$, l: $p < 0.05$ NS398 vs. DMSO; n: $p < 0.05$ NS398 vs. INDO. (77)

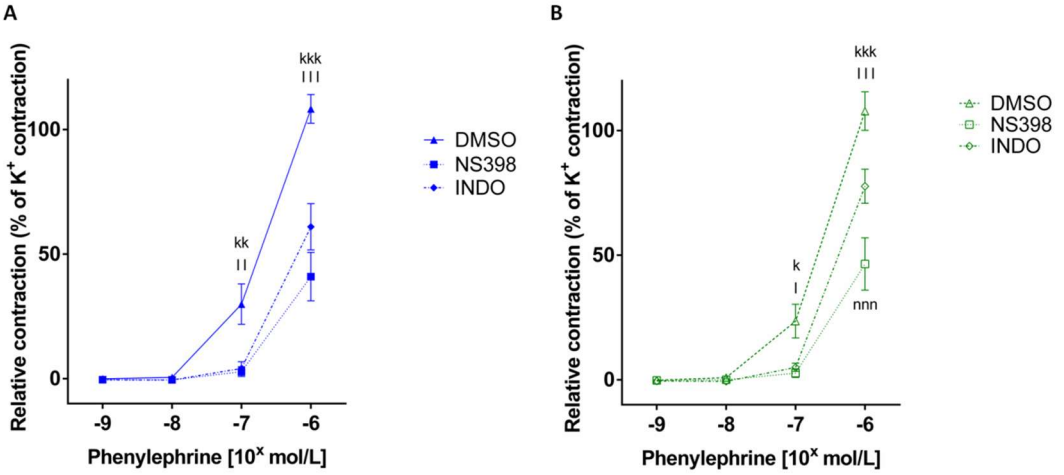

Figure 3. Phenylephrine (Phe) induced contraction in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in male Vitamin D supplemented and male Vitamin D deficient rats

A) Male Vitamin D supplemented group. In case of VD supplementation, similarly to female VD supplemented group, both general COX, and specific COX-2 inhibition led to reduced contraction with a more pronounced effect of NS398. **B) Male Vitamin D deficient group.** In male Vitamin deficient experimental group, both indomethacin and NS398 caused reduced contraction with a significantly bigger inhibition by COX-2 blocker. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented male group in blue (MD+) and VD deficient male group in green (MD–). Data are shown as mean ± SEM; N = 8 in each group; kkk: $p < 0.001$, kk: $p < 0.01$, k: $p < 0.05$ INDO vs. DMSO; lll: $p < 0.001$, ll: $p < 0.01$, l: $p < 0.05$ NS398 vs. DMSO; nnn: $p < 0.001$ NS398 vs. INDO. (77)

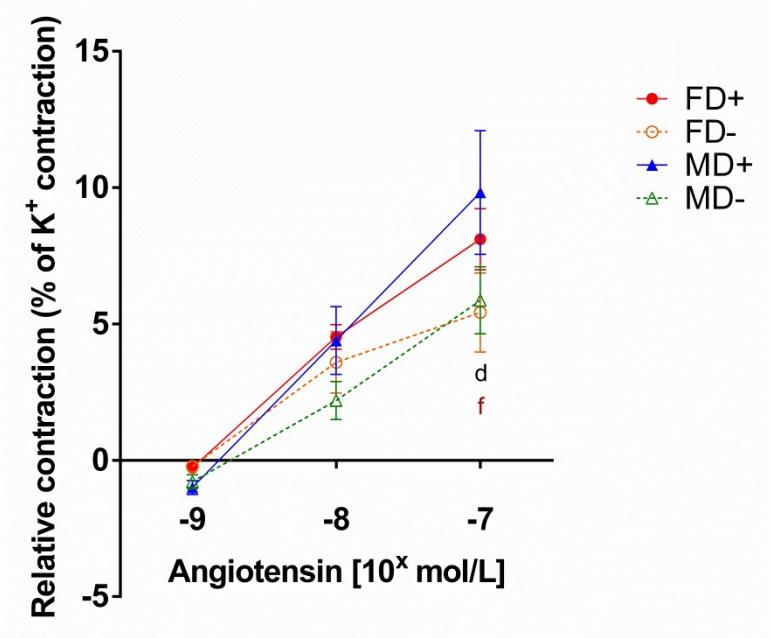

Figure 4. Angiotensin II-induced contraction of isolated renal artery segments

VD deficient male and female rats showed significantly decreased contraction at angiotensin concentration of 10^{-7} mol/L compared to VD supplemented males. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD−), VD supplemented male group in blue (MD+) and VD deficient male group in green (MD−). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status.Data are shown as mean ± SEM; N = 7-9 in each group; d: $p < 0.05$ FD−vs. MD+, f: $p < 0.05$ MD+ vs. MD−. (77)

3.1.1.4. Gender differences in relaxation of renal artery

No gender difference was observed in Ach-induced relaxation of VD supplemented group **(Figure 5)**. In the VD supplemented female group the selective COX-2 inhibitor resulted in significantly greater Ach-induced relaxation, while in the VD supplemented male group the Ach-induced relaxation was significantly increased by indomethacin, a non-selective COX inhibitor, compared to the specific COX inhibitor **(Figure 6A and 7A)**.

3.1.1.5. The effect of Vitamin D deficiency on the relaxation of renal artery

VD deficient female rats showed significantly decreased Ach-induced relaxation compared to VD supplemented females, suggesting the presence of reduced endothelial dependent relaxation mechanisms in these vessels **(Figure 5)**. In VD deficient male group, more pronounced relaxation occurred in the presence of indomethacin **(Figure 7B)**.

3.1.1.6. Gender differences in the effect of Vitamin D deficiency on renal artery

It should be emphasized as a gender difference that while the relaxation of VD deficient females was significantly reduced compared to VD supplemented females, the relaxation of VD deficient males was not significantly reduced compared to their counter group, but was also significantly reduced compared to VD supplemented females **(Figure 5)**. While in VD deficient females there was no change in relaxation in the presence of selective and general COX inhibitors, in males the relaxation increased by a general COX inhibitor, indomethacin **(Figure 6B and 7B)**.

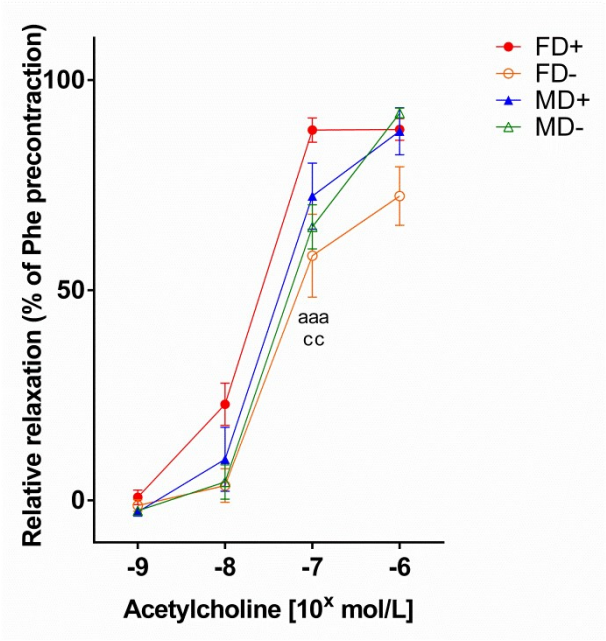

Figure 5. Acetylcholine-induced relaxation of isolated renal artery segments

VD deficient female rats showed significantly decreased relaxation at 10^{-7} mol/L compared to VD supplemented females. In addition, significantly lower relaxation was observed in male deficient group compared to the female supplemented rat at 10^{-7} mol/L Ach concentration. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD−), VD supplemented male group in blue (MD+) and VD deficient male group in green (MD−). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status.Data are shown as mean ± SEM; N = 8 in each group; **aaa**: $p < 0.001$ FD+ vs. FD−; cc: $p < 0.01$ FD+ vs. MD−. (77)

DOI:10.14753/SE.2022.2648

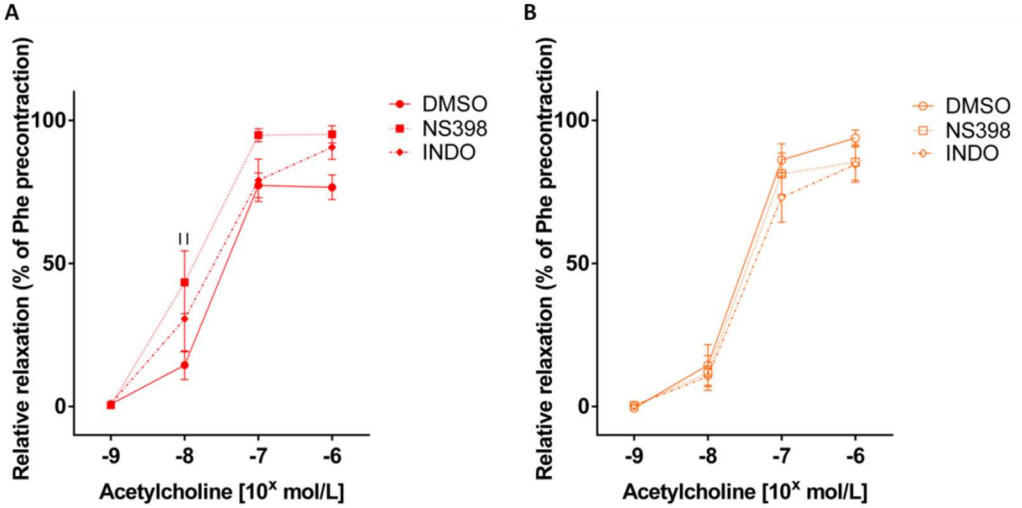

Figure 6. Ach-induced relaxation in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in female Vitamin D supplemented and female Vitamin D deficient rats

A) Female Vitamin D supplemented group. In case of Vitamin supplementation, specifix COX-2 inhibition resulted in increased relaxation; **B) Female Vitamin D deficient group.** In case of VDD, there was no significant difference in relaxation in the presence of general COX or specific COX-2 inhibitor. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD–). Data are shown as mean ± SEM; N = 7-8 in each group; ll: $p < 0.01$ NS398 vs. DMSO. (77)

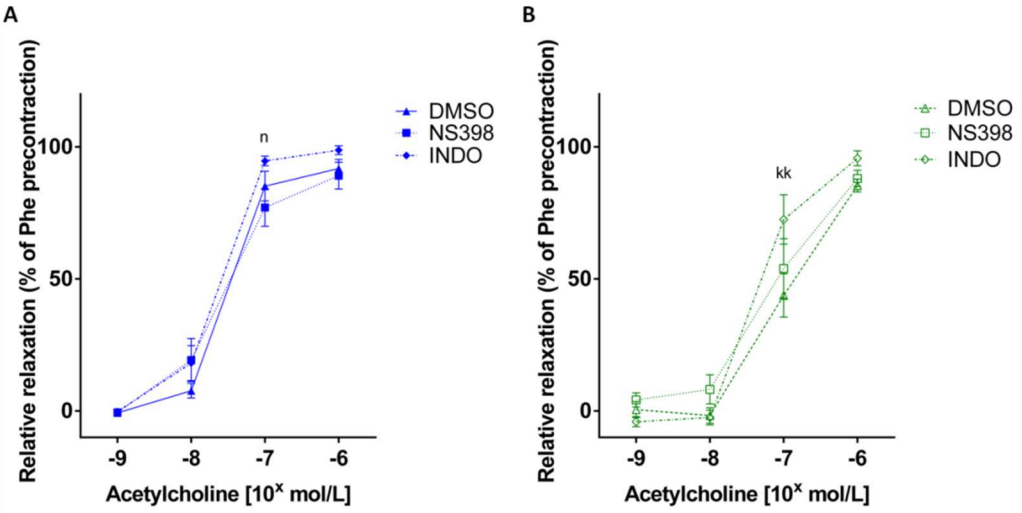

Figure 7. Ach-induced relaxation in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in male Vitamin D supplemented and male Vitamin D deficient rats

A) Male Vitamin D supplemented group. In case of Vitamin D supplemented group, general COX inhibition led to enhanced relaxation compared to specific COX-2 inhibition; **B) Male Vitamin D deficient group.** In case of VD deficient group, more pronounced relaxation occured in the presence of indomethacin. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented male group in blue (MD+) and VD deficient male group in green (MD–). Data are shown as mean ± SEM; N = 7-8 in each group; kk: $p < 0.01$ INDO vs. DMSO; n: $p < 0.05$ NS398 vs. INDO. (77)

3.1.2. Histology of Renal Arteries

The structural changes of the vessels were examined on paraffin-embedded, resorcin-fuchsin stained tissue sections. The intima/media ratio did not differ between groups **(Figure 8A)**. However, the optical density of media layer of VD deficient female animals increased significantly compared to both VD supplemented females and males ($p < 0.05$, FD+ vs. FD−and FD−vs. MD+) **(Figure 8B)**. Representative images stained with resorcin-fuchsin are shown on **Figure 8C**. However, the staining intensity of α-smooth muscle actin (α-SMA) was significantly higher in VD supplemented males compared to both female groups - that was not associated with any measured vascular function **(Figure 9A)**. Representative images of α-SMA staining are shown on Figure 9B. The smooth muscle cell nuclei density in the media layer was similar in each groups **(Figure 9C).** Representative images of this staining are shown on **Figure 9D**. The intensity of eNOS immunohistochemical staining was significantly lower in female VD deficient animals compared to VD supplemented females ($p < 0.05$, FD+ vs. FD−) **(Figure 10A).** Representative images of eNOS staining are shown on Figure 10B. Finally, the intensity of special staining of angiotensin II receptor 1 (AT1R) was significantly higher in VD deficient males than in VD deficient females **(Figure 10C)**, the representative images are shown on **Figure 10D**.

DOI:10.14753/SE.2022.2648

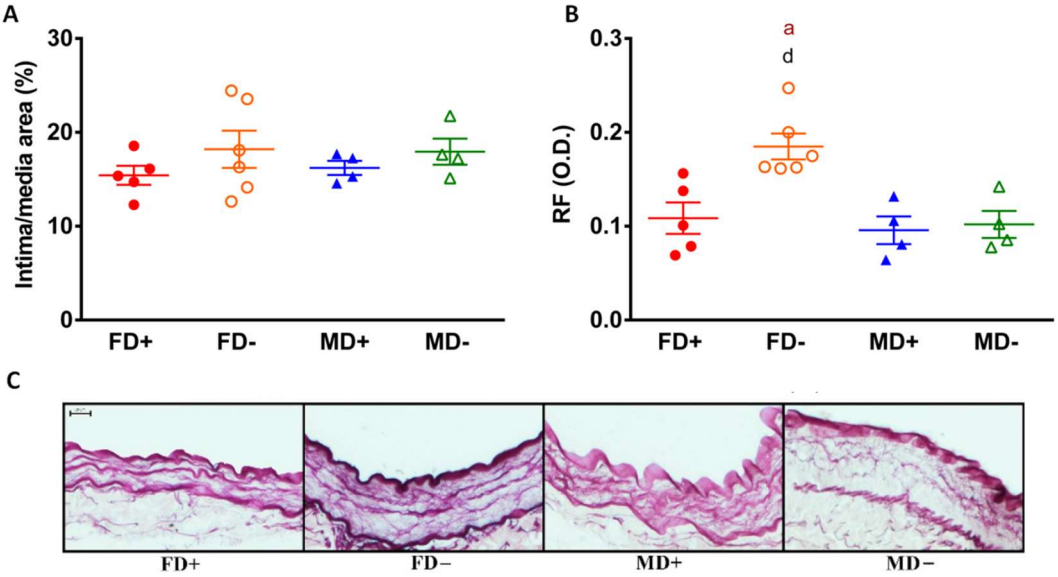

Figure 8. Resorcin-fuchsin stained renal arteries

A) Intima/media ratio of the renal arteries. There were no significant effect of VDD or gender on intima/media ratio measured on resorcin-fuchsin stained tissue sections. Neither was any intergroup difference. **B) Density of resorcin-fuchsin staining int he media layer of renal arteries.** Female Vitamin deficient group has significantly increased staining intensity compared to the female VD supplemented and male VD supplemented groups. **C) Representative images of resorcin-fuchsin stained renal arteries** (scale bar is 20 μm). Kruskal-Wallis test with Dunn's multiple comparison test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD−), VD supplemented male group in blue (MD+) and VD deficient male group in green (MD−). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status. Data are shown as median [IQR]; N = 3-6 in each group; **a**: $p < 0.05$ FD+ vs. FD−; d: $p < 0.05$ FD−vs. MD+. (77)

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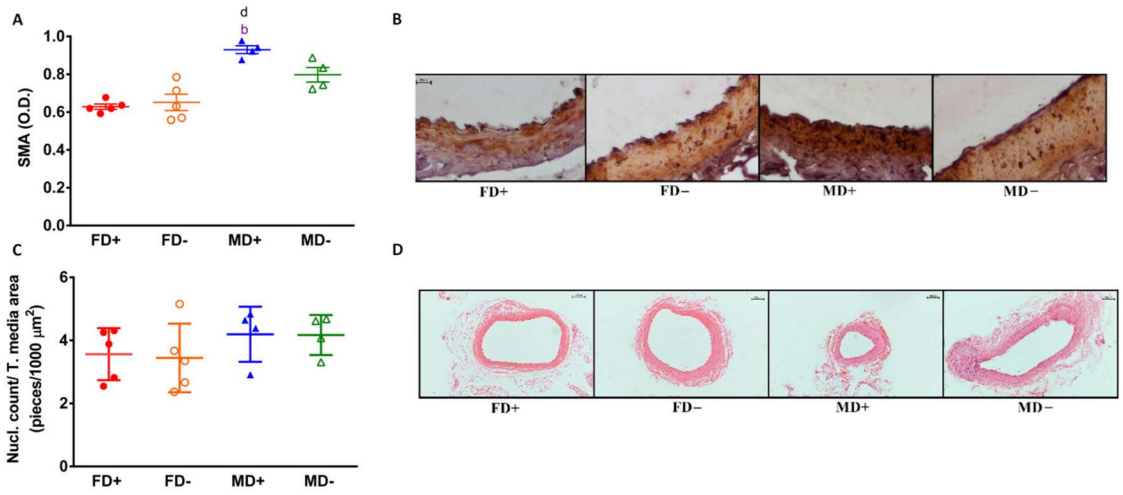

Figure 9. α-SMA staining and smooth muscle cell nuclei density of renal arteries

α-smooth muscle actin (α-SMA) immunohistochemistry of renal arteries. The VD deficient male group showed significantly higher SMA staining intensity compared to both female groups; **B) Representative images of renal arterial sections stained against α-SMA** (scale bar is 20 µm). In all immunohistochemical stainings, brown-colored diamino-benzidine represents specific labeling and blue-colored hematoxylin was used for counterstaining; **C) Density of smooth muscle cell nuclei in the media layer of renal arteries.** Between experimental groups, there were no significant difference in the number of smooth muscle nuclei per 1000 µm^2; **D) Representative images of hematoxylin-eosin stained renal arteries** (scale bar is 100 µm). Kruskal-Wallis test with Dunn's multiple comparison test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD−), VD supplemented male group in blue (MD+) and VD deficient male group in green (MD−). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status. Data are shown as median [IQR]; N = 3-6 in each group; b: $p < 0.05$ FD+ vs. MD+; d: $p < 0.05$ FD−vs. MD+. (77)

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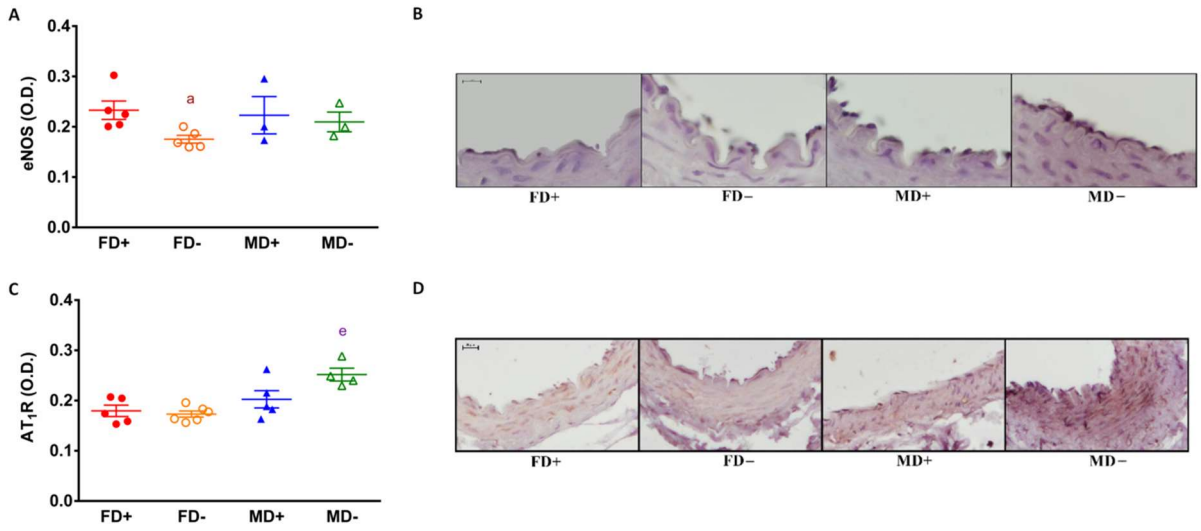

Figure 10. eNOS and AT1R immunohistochemistry of renal arteries

A) eNOS immunohistochemistry of renal arteries. eNOS specific labeling intensity was significantly lower in VD deficient female rats compared to the FD+ animals; **B) Representative images of renal arterial sections stained against eNOS** (scale bar is 10 µm); **C) Angiotensin II receptor-1 (AT_1R) immunohistochemistry of renal arteries.** AT_1R specific staining showed the highest intensity in MD− experimental group, it differed significantly from the staining intensity measured in FD− animals; **D) Representative images of renal arterial sections stained against AT_1R** (scale bar is 20 µm). Kruskal-Wallis test with Dunn's multiple comparison test by Prism 8 (GraphPad Software, San Diego, CA, USA). Each experimental group is presented in a different color; VD supplemented female group in red (FD+), VD deficient female group in orange (FD−), VD supplemented male group in blue (MD+) and VD deficient male group in green (MD−). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status. Data are shown as median [IQR]; N = 3-6 in each group; a: $p < 0.05$ FD+ vs. FD−; b: $p < 0.05$ FD+ vs. MD+; e: $p < 0.05$ FD− vs. MD−. (77)

3.2. Carotid arteries

3.2.1. Vascular Function of Carotid Arteries

3.2.1.1. Gender differences in relaxation of carotid artery

Vascular function of carotid arteries, relaxation ability of the vessels was examined by wire myograph. Gender difference in Ach-induced relaxation was found, as males showed lower relaxation level than females at 10^{-6} M acetylcholine concentration **(Figure 11A)**. VD supplemented females showed significantly higher relaxation than VD supplemented males (and also than the VD deficient males) **(Figure 11B)**. No difference was seen in the inhibitory effect of L-NAME on relaxation and in the effect of L-NAME + INDO between groups, whereas indomethacin alone increased endothelium-dependent relaxation significantly in the supplemented male group **(Figure 12A and 13A)**.

3.2.1.2. The effect of Vitamin D deficiency on relaxation

VDD reduced the level of relaxation in both sexes at 10-6 mol/L Acetylcholine concentration **(Figure 11A and 11B)**. In case of L NAME, INDO and co-incubation VDD was not resulted in significant difference between groups **(Figure 12B and 13B)**.

3.1.1.3. Gender differences in the effect of Vitamin D deficiency

No gender difference was found in VD deficient groups, VD deficient females only differed from VD deficient male counterparts **(Figure 11B)**. Furthermore, like in VD supplemented males, indomethacin alone increased endothelium-dependent relaxation in VD deficient males, whereas such effect was not observed in VD deficient females **(Figure 12B and 13B)**.

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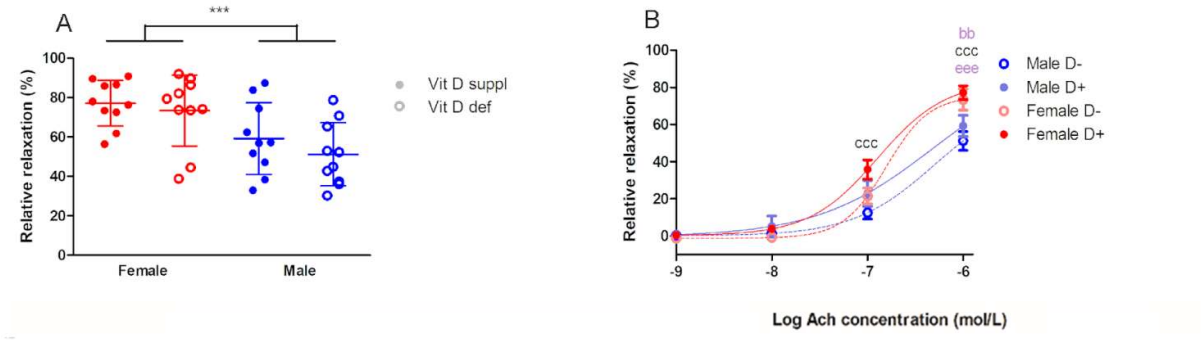

Figure 11. Acethylcholine (Ach) induced relaxation of carotid arteries

A) Ach-induced relaxation in the four 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 represents mean±SD. N = 9-11 in each group; two-way ANOVA; factors: gender, VD status. ***: p<0.001 test by Prism 8 (GraphPad Software, San Diego, A, USA). **B) Ach-induced relaxation.** Male rats showed significantly reduced relaxation compared to females at Ach concentration of 10^{-6} mol/L independently from VD status. Data are shown as mean±SEM**;** N=9-11 in each group; repeated measures ANOVA, Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Gender difference is highlighted by violet, while burgundy color indicates significant difference due to different VD status; bb: p < 0.01 FD+ vs. MD+; ccc: p < 0.001 FD+ vs. MD−; eee: p < 0.001 FD−vs. MD−; (78)

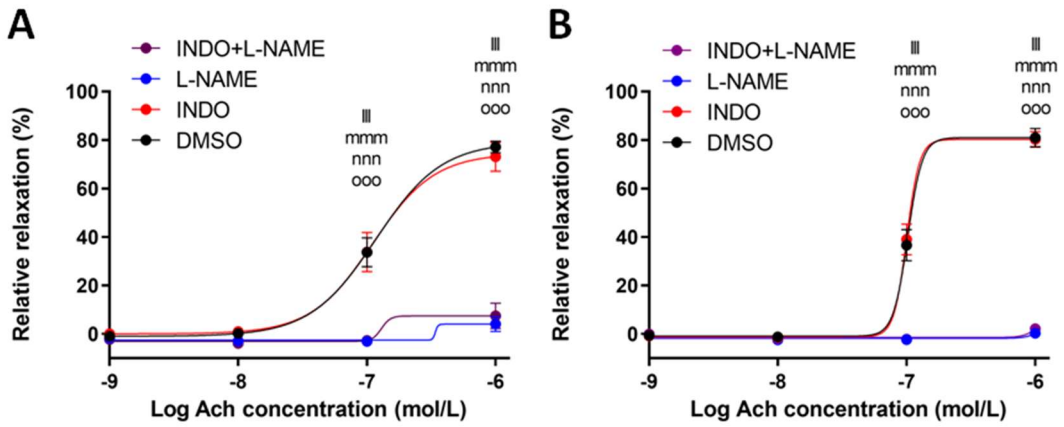

Figure 12. Ach-induces relaxation in the presence of nitro-L-arginine methyl ester or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in female Vitamin D supplemented and female Vitamin D deficient rats

A) Female Vitamin D supplemented group. B) Female Vitamin D deficient group. L-NAME blocked the vasodilation in female VD supplemented and deficient groups, co-incubation with INDO had no additional effect. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Data are shown as mean ± SEM; N = 6-11 in each group; lll: p < 0.001 DMSO vs. L-NAME; mmm: p < 0.001 DMSO vs. INDO+L-NAME; nnn: p < 0.001 INDO vs. L-NAME; ooo: p < 0.001 INDO vs. INDO+L-NAME (78)

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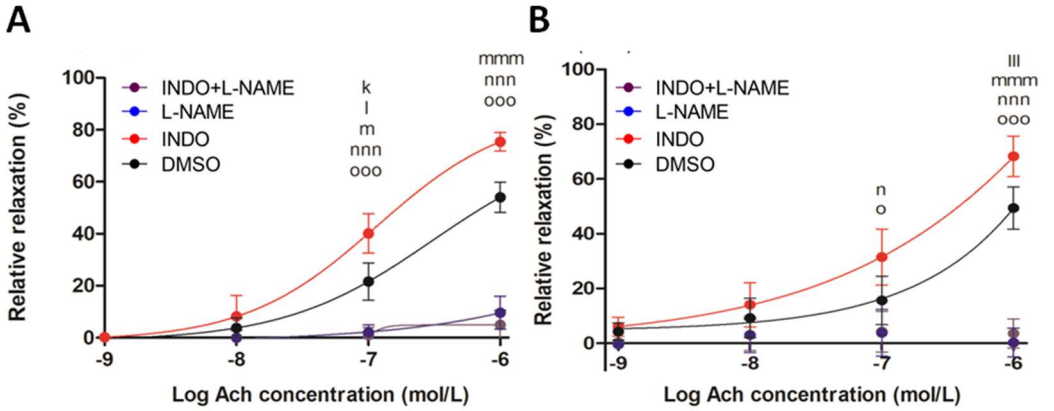

Figure 13. Ach-induces relaxation in the presence of nitro-L-arginine methyl ester or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in male Vitamin D supplemented and male Vitamin D deficient rats

A) Male Vitamin D supplemented group. B) Male Vitamin D deficient group. L-NAME blocked the vasodilation in male vitamin D supplemented and deficient groups, co-incubation with INDO had no additional effect. INDO itself increased the degree of relaxation only in both male groups. Repeated measures two-way ANOVA with Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). Data are shown as mean ± SEM; N = 6-11 in each group; k: $p < 0.05$ DMSO vs. INDO, l: $p < 0.05$ DMSO vs. L-NAME; lll: $p < 0.001$ DMSO vs. L_NAME; m: $p < 0.05$ DMSO vs. INDO+L-NAME; mmm: $p < 0.001$ DMSO vs. INDO+L-NAME; n: $p < 0.05$ INDO vs. 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 (78)

3.2.2. Histology of Carotid Arteries

After deparaffinization, eNOS immunohistochemical staining density was significantly higher in the FD+ group compared to the MD- group on paraffin-embedded sections **(Figure 14)**.

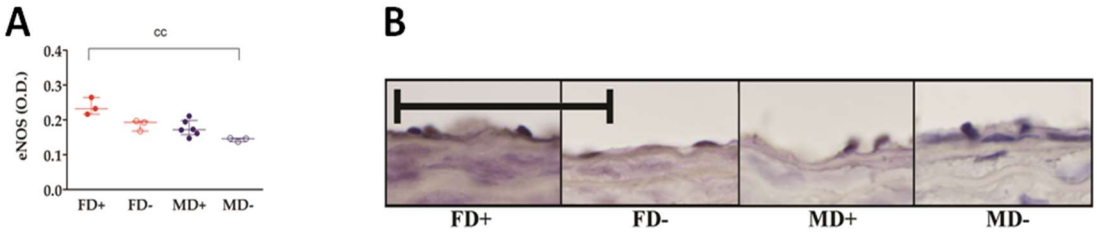

Figure 14. eNOS immunohistochemistry of carotid arteries

A) Optical density of eNOS labeling int he intimal layer of carotid arteries. eNOS specific labeling intensity was significantly lower in VD deficient males; **B) Representative images of carotid arterial sections stained against eNOS** (scale bar is 10 µm). Kruskal-Wallis test with Dunn's multiple comparison test by Prism 8 (GraphPad Software, San Diego, CA, USA). Data are shown as median [IQR]; N = 3-6 in each group; cc: $p < 0.01$ FD+ vs. MD− (78).

DOI:10.14753/SE.2022.2648

4. DISCUSSION

Recently one of the most significant health problems is cardiovascular (CV) morbidity and mortality, which is still the leading cause of death worldwide. The renal and carotid arteries are of major importance for cardiovascular disease. The renal arteries play a role in the pathomechanism of hypertension, the carotid arteries are involved in the development of stroke. It has been known for a long time that there is a significant difference in the development and severity of cardiovascular disease between men and women to the benefit of women that disappears after menopause. Furthermore, VDD is common, but it is also involved in the development and severity of many diseases, while its supplementation is beneficial for most of our organs. Gender differences in the effects of VD are also suggested.

Our findings indicate that VDD increases the cardiovascular risk in both sexes.

Relaxation disfunction was found in both males and females as a result of VDD, but significant gender differences were also observed in contraction and relaxation mechanisms.

4.1. Contractile changes in response to Vitamin D supplementation and deficiency on the renal arteries of male and female rats

In our recent study no gender difference was found between VD supplemented groups in Phe-induced contraction, generalized COX inhibition, specific COX-2 inhibition and AII induced contraction. (13). However, it is important to consider that gender differences in vasoconstriction and vasodilation can be also affected by the type of the vessels. Gender differences in vasoconstriction can be observed in mesenteric, coronary, or carotid arteries (5, 6, 13). In mesenterial artery rings the release of cyclooxygenase derived constrictor factors is more intense in male than in female SHR animals (5). Furthermore, serotonin induced vasoconstriction on carotid arteries of mice is significantly higher in male than in female animals (2). In resistance coronary arteries male rats are significantly more sensitive to endothelin-1 vasoconstrictor effect than females (6). Also in resistance coronary arteries the myogenic tone and response to thromboxane agonist is higher in

males compared to female animals (7). In contrast, no gender difference was observed in endothelial dependent vasoconstriction of popliteal artery (8).

However, significant gender difference was found in the contractile response of renal arteries to VDD. In the renal arteries of female rats the phenylephrine induced contraction increased significantly, while in males the phenylephrine induced contraction remained unchanged. It is well reported in the literature that VDD increases cardiovascular risk, and in addition to the traditional cardiovascular risk factors, VDD is an independent predictor of the severity of coronary diseases (including the level of stenosis, the number of the affected vessels as well as the degree of coronary calcification (79)). The adverse effect of VD on cardiovascular risk factors can be associated with the significant increase of arterial stiffness that occurs in its absence, which is dose-dependently reduced by VD supplementation both in men and women (25, 79). Furthermore, VD modulates directly the endothelium-related contraction response in a calcium-dependent way by reducing calcium influx into endothelial cells, thereby reducing the amount of endothelium-derived contraction factors in aortic rings (81), i.e. in the absence of VD the contraction response can be enhanced, as it was observed in case of female animals in our recent study.

In contrast to Phe-induced contraction, where a significant increase was observed in contraction in response to VDD – angiotensin II induced contraction was reduced in the presence of VDD in both groups compared to VD supplemented males. The most intense contraction was observed in VD supplemented male animals. As a result of our α-SMA immunohistochemistry staining, the smooth muscle actin was the highest in VD supplemented males compared to female groups. This can be the consequence of the fact that androgen hormones increase the proliferation of human vascular smooth muscle cells (50), while oestrogens inhibit both the proliferation and the migration of human vascular smooth muscle cells regardless of gender (55). Furthermore sexual steroids affect also the vascular sensitivity of angiotensin II, as it is increased by testosterone and reduced by oestrogen (51). The observed gender difference in the volume of smooth muscles may provide an explanation for the highest angiotensin II-induced contraction in VD supplemented males. However, compared to female groups, there was no significant difference either in AII induced contraction, or in α-SMA immunohistochemistry staining in males in the presence of VDD. It leads to the conclusion that there is no gender difference in the effect of VDD on smooth muscle cells. The most intense angiotensin II

receptor type 1 (AT1) staining was observed in VD deficient male animals. This can be the result of the fact that renin-angiotensin-aldosterone system (RAAS) is influenced by both the sex hormones and VD. Both oestrogen and VD reduce the expression of AT1 receptor (28, 56), moreover oestrogen reduces the synthesis of angiotensin converting enzyme (51). In contrast, testosterone and VDD increases the activity of RAAS system (51, 58). The lowest optical density value was measured in VD deficient females, which can be explained by the lower smooth muscle element content of these animals, that can also affect the amount of the receptors by tissue unit.

The inhibition of prostanoid pathway by selective COX-2 inhibitor, NS398 and non-selective COX inhibitor, indomethacin reduced the phenylephrine induced vasoconstriction in VD supplemented and also in VD deficient male and female animals. As a gender difference it should be emphasized that in males COX-2 had a significantly more intense inhibition compared to indomethacin, whereas such effect could not be observed in females. This can be the result of the fact that COX-2 inhibiting vasoconstrictor inhibits the prostanoid pathway via thromboxane-prostanoid (TP) receptors in VD deficient males. TP receptor agonists, including thromboxane A_2 and other vasoconstrictive prostanoids, such as 20-hydroxyeicosatetraenoic acid (20-HETE), contribute to vascular dysfunction, vascular smooth muscle proliferation (82). Indomethacin, as a non-selective COX inhibitor, has both COX-1 and COX-2 inhibitory effects; the less intense contraction compared to COX-2 can be a result of that a weak relaxation effect via COX-1 is also inhibited, as the summed effect is a more specific blockade of the contractile effect compared to a selective COX-2 inhibitor. An animal study suggested, that VD is able to decrease COX-2 expression; in oestrogen deficiency calcitriol therapy reduces the overexpression of COX-2 and thromboxane prostanoid (TP) receptor in renal arteries and aortic endothelial cells (83). Furthermore, in prostate cancer cells VD therapy reduces mRNA and protein level and expression of COX-2 (84, 85). Interestingly, VD down-regulating effect is specific to COX-2, there is not such effect on COX-1 (82) A possible mechanism of the adverse effects of androgens on cardiovascular system can be that the androgen receptor agonist, dihydrotestosterone (DHT) increases the vascular inflammatory mediator COX-2 in rodent cerebral arteries and human coronary artery smooth muscle cells independent from an inflammatory stimulus and such effect is androgen-dependent. The effect of DHT on COX-2 contains both androgen

receptor-dependent and –independent mechanisms (86). Furthermore, in male rats chronic testosterone therapy increases COX-2 level in cerebral vessels of male rats, whereas 17-beta-oestradiol therapy significantly reduced cerebrovascular level of COX-2 in male rats. These data suggest that androgens can make the vessels more vulnerable by enhancing inflammatory processes (87). Thus, in male rats, the presence of testosterone and its interaction with VDD may have resulted in increased COX-2 response.

4.2. Relaxation changes in renal and carotid arteries of male and female rats in the presence of Vitamin D supplementation and deficiency

4.2.1. Renal arteries

Regarding relaxation, as in vasoconstriction, gender differences can be observed depending on the type of the investigated vessels. FMD on brachial artery doesn't differ between men and women, while FMD of popliteal artery is significantly higher in physically active adult women (88). Non-endothel mediated dilatation (nitroglycerin) is also lower in men's brachial artery (9). No gender differences were found in acetylcholine-induced relaxation of renal arteries in VD supplemented groups. Ach-induced relaxation was decreased in VDD females. The observed increased contractility and reduced acetylcholine-induced relaxation was accompanied by the decrease in the intensity of eNOS immunohistochemical staining, that shows the developing of endothel dysfunction in these animals. Ach-induced relaxation in renal arteries was lower in males compared to VD supplemented females, that may indicate the enhanced vulnerability of blood vessels in male animals to certain damaging agents such as VDD. However, no difference in intensity of eNOS staining was found in renal arteries in males. Possibly, the suboptimal VD level interferes with the transcription or activity of endothelial enzymes, despite eNOS staining intensity is unaltered in male animals, but the bioavailability of NO is decreased, may be the result of elevated oxidative stress or a limited eNOS phosphorylation. Our study team has previously shown the relaxation-reducing effect of VDD on resistance coronary arteries (27). The positive effect of VD on vascular relaxation is confirmed by the observation that both in vitro and in vivo VD supplementation increase acetylcholine-induced relaxation (28, 30). This positive effect

of VD on relaxation is achieved by increasing eNOS expression and NO production (28-30). The phenomenon of increased intensity of eNOS staining is absent in VDD (89, 90), this reduced intensity of eNOS straining refers to the deterioration of NO-dependent relaxation mechanisms. Although no differences in eNOS intensity was observed in the renal rings of male animals, the expression of the enzyme was not altered, and the reduced NO bioavailability may have played a role in the lower relaxation capacity seen in males. The increased level of reactive oxygen radicals can reduce the bioavailability of NO, as the formed superoxide spontaneously reacts with nitrogen monoxide to create peroxynitrite, thus reducing its concentration. Peroxynitrite itself has an oxidative effect, which further impairs vascular function (91, 92). Besides being an antioxidant itself (93), VD reduces the level of peroxynitrite (94), so the reduced relaxation observed in renal rings of VD deficient males can be a consequence of reduced NO bioavailability.

Ach-induced relaxation was investigated also by adding selective COX-2 and non-selective COX inhibitors, that had diverse effect on acetylcholine-induced relaxation in different sex. Selective COX-2 inhibitor increased Ach-induced relaxation in VD supplemented females. Both in VD supplemented and deficient males' relaxation was increased by non-selective COX inhibitors. In VD supplemented males relaxation was significantly increased by non-selective COX inhibitors compared to selective COX inhibitors, while in case of VD deficient males a more pronounced relaxation occurred in the present of indomethacin compared to its vehicle. In females more intense inhibitory effect of COX-2 was observed, while in males, a non-selective COX inhibitor, indomethacin had more a pronounced impact. This could be explained by the fact that oestrogen increases COX-2 expression (95, 96), leading to the increased impact of COX-2-related processes in females. Reduced contraction and increased relaxation in the presence of COX inhibitors suggest a COX-inhibition caused absence of a vasoconstrictor mediator. The compounds produced by the cyclooxygenase enzyme can have both vasoconstrictor and vasodilator effects: endoperoxides derived from arachidonic acid, such as prostaglandin (PGH2), have smooth muscle contractile effects. Endoperoxides can be further converted by their own synthases to prostacyclin (PGI2), thromboxane A2 (TXA2), prostaglandin D2, prostaglandin E2, prostaglandin F2, etc.; from which prostacyclin synthase is dominant in the endothelium (97), PGI2 causes vasorelaxation by acting on its own receptor (98). COX-1 and COX-2 play an important role in producing

so called endothelium-derived contracting factors (endothelium-derived contracting factors, EDCF). Substances, such PGH2, TXA2 and other prostanoids and prostacyclins, that cause vasoconstriction via the thromboxane-prostanoid (TP) receptor. The level of these factors increases in several pathological processes such as hypertension or diabetes (97, 99, 100). The connection between VD, cyclooxygenase-derived factors and the function of renal arteries is also confirmed by a study, in which VD therapy restores the impaired endothel-dependent relaxation of renal arteries by decreasing the expression of COX-2 and TP receptors in oestrogen deficient female rats (83).

4.2.2. Carotid arteries

In contrast to renal arteries, lower relaxation was observed in the carotid arteries of males than of females independently form VD status. Non-endothel dilation (nitroglycerine mediated dilation) was also lower in men's brachial artery (9).

1,25(OH)2D3 induces vasodilation, relieves arterial pressure and it can improve blood flow after stroke by potentiating NOS and reducing the level of vasoconstriction via inhibition of the renin-angiotensin axis (30). In our recent study – as we did also in our previous studies −we observed decreased relaxation ability in both sexes. More intense vasorelaxation was found in female groups, especially in VD supplemented females. Obvious gender difference appears in Ach relaxation, which was slightly mitigated by VDD at lower concentrations, but it was maintained at maximum concentration.

Endothelium-dependent vasodilation is also highly influenced by gender, leading to higher cardiovascular risk of men. Sex hormone induced expression of enzymes involved in the synthesis of endothelium-derived relaxation factors, like eNOS, COX-1 and 2, and prostacyclin synthase is beleived to play role in this phenomenon (101). It is widely accepted in the literature that estrogen has a determining role in the regulation of the brain vascular contractility function by enhancing the production of anti-thrombotic cyclooxygenase-1 (COX-1)-derived substance and the prostaglandin I2 (PGI2), acting as strong vasodilatators. Therefore, in women in the premenopausal period, estrogen may provide an increased protection against the development of cardiovascular diseases, including stroke (102).

When examining relaxation, eNOS expression showed both gender- and VD related differences, i.e. lower expression was found in males and in VDD, which was the lowest

in VD deficient males. The production of oxygen derived free radicals, especially superoxide can reduce the bioavailability of nitric oxide through their spontaneous reaction creating the potent oxidant peroxynitrite (36). The reduced relaxation in males can be the result of a COX-dependent vasoconstrictor effect, which is detectable in males independently from the presence of VD, that can not be observed in females. Upon the findings of previous studies gender difference may be supposed in COX function (102, 103). Release of cyclooxygenase constrictor factors on mesenteric arterial rings is stronger in male SHR animals, than in female SHRs (5). In aortic rings, acetylcholine-induced relaxation is significantly greater in SHR female animals compared to males, with a parallel increase in relaxation in males and without any changes in young females as a response to INDO inhibition. In parallel, significantly lower COX-1 protein level was found in young, 16-weeks-old female SHR rats compared to males (103). However, no difference was seen in the intensity of eNOS staining in the renal artery of males, whereas eNOS expression showed both sex- and VD related differences in males, i.e. lower expression was found in males and in VDD, which was the lowest in Vitamin deficient males. We have discussed above the connection between eNOS and VD in details. Gender differences in eNOS expression has already been stated by others: expression level of eNOS on aortic rings was reported higher in females than it was in males (104).

No difference in L-NAME effect was observed between males and females, nor in the effect of VDD, which was not even influenced by co-incubation with INDO. However, INDO alone significantly increased the relaxation level in both male groups. A possible explanation could be that indomethacin, as a non-selective COX inhibitor inhibited the constrictor prostanoid pathway in males. As it has been discussed previously, gender difference was found in COX-2 level, as testosterone increases the level of COX-2, while oestrogen has no such effect (86, 87). Furthermore, activation of COX (most likely COX-1)-derived prostanoids and activation of thromboxane receptors counteracts Ach-induced relaxation in mesenteric vessels of male mice compared to females, probably it is due to the increased smooth muscle depolarization of the arterial wall of male mice (105).

4.3. Morphological alteration in response to Vitamin D supplementation and deficiency in renal arteries of male and female rats

We examined structural changes of renal arteries on resorcin-fuchsin stained sections. Although the ratio of tunica intima to media thickness did not change, elastic fiber density increased in the female VD deficient group, which may have contributed to the increased contractile capacity and decreased relaxation ability; that can be the first sign of a more rigid vascular wall. Upon literature data we can state that elasticity of the aorta impairs due to VDD (106). Furthermore, $1,25(OH)_2D_3$ is inversely correlated with vascular calcification: low serum $1,25(OH)_2D_3$ level leads to more severe coronary artery calcification in patients with coronary heart disease (107). In our recent study not any morphological differences were found on renal arteries of VD deficient males, whereas tunica media thickened in cerebral arteries, intima/media ratio reduced and the vascular lumen narrowed in VD deficient male rats (35). As these changes also occurred in females when hyperandrogen status accompanied by VDD, that suggest a possible gender specific regional difference in vascular remodeling induced by VDD.

4.4. Possible explanations of gender differences in Vitamin D deficiency

The main gender differences in our recent study are that in females the contractor/relaxation balance shifted towards contraction due to the presence of VDD in case of Phe-induced contraction and reduced Ach-induced relaxation in renal arteries, while only reduced Ach-induced relaxation could be observed in the renal arteries of male rats, increased Phe-induced contraction was not present. Males had a lower relaxation capacity in their carotid arteries compared to females in case of both VD supplementation and deficiency. Thus 8-week-long VDD in young healthy rats of both sexes leads to early impairment of vessel function in case of renal as well as carotid arteries that increases the cardiovascular risk later in life.

It is important to emphasize that the observed gender differences were not generated from body size variations or difference in lifestyles, as our animals received VD supplementation on a weight-based during the chronic treatment of our rat model. Weight-based supplementation resulted in lower $25(OH)D_3$ level in males compared to females (33, 35). Previous studies established that VD level is lower in men than in women and that the prevalence of VDD is higher in males (108-111). In these experimental models VD supplementation was not normalized, so the measured difference could be the result of body weight variations or lifestyle differences. However

in our own rat model VD supplementation was adjusted to body weight, so the observed gender difference could not be related to differences of body weight or lifestyles. The found difference in VD levels can support the proposal that there are gender differences in the bioavailability, absorption and metabolism of oral VD supplementation. That can happen as sex hormones affect the VD level of the body. In the presence of testosterone deficiency $25(OH)D_3$, VDBP and $1,25(OH)_2D_3$ serum level decreases, which can be restored by testosterone treatment, while the expression of VDBP is increased by testosterone in orchidectomized male rats (112). While in female rats ovariectomy reduces the level of 1,25-dihydroxyvitamin D, that can be restored by oestrogen supplementation; however, ovariectomy and oestrogen supplementation did not have a significant effect on VD binding protein (113). Lee et al found that DHT induces 25-hydroxyvitamin D3-1α hydroxylase CYP27B1 and obstructs 25-hydroxylase enzymes CYP24A1. CYP27B1 that converts 25-OH-Vitamin D3 to calcitriol. When male kidneys preferentially hydroxylate $25(OH)D_3$ using 24-hydroxylase rather than $25(OH)D_3$-1-alpha hydroxylase, DHT suppressed the progesterone-receptor-mediated 24-hydroxylase expression, and it is important to note that DHT increased the blood $25(OH)D_3$ levels. These findings uncover an important link between androgens and vitamin D homeostasis and suggest that therapeutic modulation of progesterone may be used to treat vitamin D deficiency and related disorders. CYP24A1 codes another hydroxylase that converts calcitriol into an excretable form. These mechanisms both leads to lower serum VD levels even though we have not seen its deteriorating effect (114).

The literature provides contradictory results concerning the cardiovascular risk increasing effect of VD. Upon a recent meta-analysis based on human trials VD supplementation did not show beneficial effect on cardiovascular risk (115). However, studies on VDD and supplementation need to be approached critically, paying attention to differences between the individual studies; e.g. not taking optimal level of VD supplementation is a common mistake. In the above mentioned meta-analyses the average age of the included subjects was 66 years, and most of these studies involved solely those patients who had an already existing cardiovascular disease. In human studies, VD was a proposed treatment to preexisting conditions while our VD supplementation rather serves a preventive measure. Furthermore, it is really difficult to separate VD supplemented and deficient subgroups in human trials. The unknown duration of previous hypovitaminosis

can also be a limitation of these studies. However, other studies described an increased cardiovascular risk associated with VDD, and in addition to the traditional cardiovascular risk factors, VDD is considered to be an independent predictor of the severity of coronary heart disease (25, 78, 79, 116).

A possible explanation of the observed gender differences in VD deficient groups can be the presence of an interaction between sex hormones and VD, that is also confirmed by human and animal study results. Although, the prevalence of VDD is higher in males, females are more sensitive to VDD. A Swiss cohort study found an inverse relation between cardiovascular mortality and 25(OH)D_3 levels in women (mean age at baseline was 47.1 years), while this inverse correlation was missing in males (117). Furthermore, the prospective Monitoring of Trends and Determinants of Cardiovascular Disease (MONICA) study reported a higher cardiovascular mortality in VDD females compared to males (118). In vitro experiments show that VD modifies the effects of testosterone and oestrogen in vascular smooth muscle and endothelial cells in a dose- and cell-specific manner. VD probably achieves such effect by increasing the expression of vascular oestrogen receptor (119). One of the connections between oestrogen and VD is that they mutually increase the expression of each other's receptors: oestrogen analogs increase the expression of VDR in human umbilical artery vascular smooth muscle cells (119, 120); while VD analogs increase the expression of oestrogen receptors (120, 121). Interestingly VD also increases the expression of androgen receptors (AR), but testosterone, unlike oestrogen, has no increasing effect on the expression of VDR (122, 123). Vitamin D also affects aromatase (the enzyme responsible for converting testosterone to estrogen) expression: in a mouse model, ovarian and testicular aromatase activity and expression were decreased in VDR knockout animals, while their serum estrogen levels were lower also (124). These findings suggest that the sensitivity of blood vessels to the effect of oestrogen and VD can be mutually enhanced by oestrogen and VD, and may even enhance the vasoprotective effects of each other's (121). It is possible that protective effects of oestrogen are impaired in VDD, that can contribute to women's increased sensitivity to VDD .

DOI:10.14753/SE.2022.2648

5. CONCLUSIONS

Our experiments focused on the following questions:

1) Are there gender differences in renal and carotid artery function in Vitamin D supplemented young adult male and female rats?

No gender differences were found in Phe-induced and AII-induced contraction and in Ach-induced relaxation in renal arteries of Vitamin D-treated rats. However, whereas the specific COX-2 inhibitor significantly increased Ach-induced relaxation compared to vehiculum in vitamin D-treated females, the general COX inhibitor, indomethacin significantly increased Ach-induced relaxation compared to the inhibitory effect of specific COX-2 in Vitamin D-treated males. A significant gender difference was found in Ach-induced relaxation in carotid rings, as females had more intense relaxation compared to males.

2) Are these attributes altered by Vitamin D deficiency?

Maladaptive changes occurred in vessel functions due to VDD. Phe-induced contraction level increased significantly in females' renal arteries. General and special COX-2 inhibition pre-treatment significantly decreased the contraction level in both sexes. Furthermore, the level of Ach-induced relaxation decreased significantly in renal arteries of both sexes. In parallel with these findings, our histological results demonstrated that optical density of media layer of female animals increased significantly compared to VD supplemented females and males, that may have contributed to the increased contractile force and the reduced relaxation ability of these vessels. Furthermore, also in VD deficient females, the development of endothel disfunction was indicated by the reduced intensity of eNOS staining. The level of relaxation decreased in carotid arteries in both sexes as a result of VDD.

3) Are there gender differences in the lesions caused by vitamin D deficiency?

Among gender differences in the vascular effects of VDD it is worth highlighting that vascular response was shifted towards vasoconstriction in renal vessels of female animals, as the Phe-induced contraction increased, while Ach-induced relaxation decreased. In males only reduced relaxation was observed, the increased constrictor response was

missing. In contrast, no gender difference was detected in carotid arteries between VD deficient groups.

DOI:10.14753/SE.2022.2648

6. SUMMARY

Functional adaptation and gender differences in renal and carotid arterial rings in a pet model of VDD and supplementation have not been studied in detail yet. Therefore, we aimed to explore the adaptations of contraction and relaxation of such arteries to VDD and supplementation in male and female rats. Wistar rats were separated into 4 groups such as female VD supplemented, female VD deficient, male VD supplemented and male VD deficient groups. The treatment lasted 8 weeks, VDD was initiated with a Vitamin D-free diet, while the VD supplemented groups received additional VD supplementation in combination with the conventional rat diet. After having applied a chronic treatment, we examined the functional characteristics of renal and carotid vessels by wire myograph. VDD in isolated renal arteries of female rats showed increased Phe-induced contraction. Phe-induced contraction was reduced by both indomethacine and selective COX-2 inhibitor in all study groups. AII-induced contraction was the highest in Vitamin-D supplemented males. Acetylcholine-induced relaxation impaired in both VD deficient groups. NS398 in VD supplemented female animals and indomethacin in males reduced Ach-induced relaxation better. Increased elastic fiber density was observed in VD deficient females. The density of AT1R staining was the highest in VD deficient male group. VDD led to vascular disfunction in renal arteries in both genders, but in females more extensive impairment developed that was accompanied by enzymatic and structural changes. Gender differences can be observed in Ach-induced relaxation of carotid vessel rings, VD supplemented females showed significantly higher relaxation compared to males. In presence of VDD the level of relaxation reduced in both sexes.

As a result of VDD vascular impairment developed in renal and carotid arteries of both sexes. But in pathomechanism gender differences can be supposed. Thus, in line with the literature it can be stated that VDD increases cardiovascular risk and VD is supposed to have a cardioprotective effect.

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Publications not related to the thesis:

Stark J, Várbíró Sz, Miklós S, Tulassay Zs, Sára L, Adler I, Dinya E, Magyar Z, Székács B, Marczell I, Kloosterboer HJ, Rácz K, Békési Z. Antioxidant effect of the active metabolites of tibolone. Gynecol Endrocinol. 2015
IF: 1,413

∑IF: 13.053

DOI:10.14753/SE.2022.2648

9. ACKNOWLEDGEMENTS

Thorough the whole process leading this dissertation I have received indispensable support and assistance.

First I would like to thank my supervisor Prof. Szabolcs Várbíró DSc and my co-supervisor Dr. Eszter Horváth PhD for their careful guidance and continuous incentive from the planning of the study until to the presentation of this thesis. This was a 6-year long hard work for all of us, trying developing me – a born clinician – to a researcher. Based on these I have changed to a clinician who is looking for scientific evidences in the background of clinical intuitions and experiences and if not satisfied, ready to research them.

Life long gratitude to Dr. Mária Szekeres PhD and Dr. György Nádasy PhD, who introduced me the complex methodology of research and the wire-myography. Less, than 100% of precision in the research and it's documentation was never accepted.

I would like to thank to my research fellows and colleagues, working together in the research, in the data collection and analysis and in writing the publications which were accepted in highly ranked D1 scientific papers confirming the actuality and quality of the study. Bálint Bányai, Hicham Dalloul, Leila Hadjadj, Dóra Gerszi, Judit Kiss, Réka Kollarics, Péter Magyar, Borbála Péterffy, Eszter Soltész-Katona, Anita Süli, Réka Eszter Sziva, Marianna Török.

I would like to thank Dr. Rita Benkő for overhauling my dissertation with a perfectionism of a scientist and as a first reviewer she enriched the it with many valuable thoughts.

And finally I have to thank my colleagues and my family. During the time we performed the measurements, collected and analyzed the data, wrote the articles and the dissertation, there could have been a lot of conversation, playing, hiking, or just being together. This dissertation also glorifies their endless acceptance and patience.