Vascular Pharmacology 122–123 (2019) 106581

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Vitamin K_2-MK-7 improves nitric oxide-dependent endothelial function in ApoE/LDLR$^{-/-}$ mice

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ARTICLE INFO

Keywords:
Endothelial function
Atherosclerosis
MRI
Menaquinone-7

ABSTRACT

Although, vitamin K_2 displays vasoprotective effects, it is still not known whether K_2 treatment improves endothelial function.

In ApoE/LDLR$^{-/-}$ mice at the stage prior to atherosclerosis development, four-week treatment with K_2-MK-7, given at a low dose (0.05 mg/kg), improved acetylcholine- and flow-induced, endothelium-dependent vasodilation in aorta or in femoral artery, as assessed by MRI *in vivo*. This effect was associated with an increased NO production, as evidenced by EPR measurements in *ex vivo* aorta. Treatment with higher doses of K_2-MK-7 (0.5; 5 mg/kg) resulted in a dose-dependent increase in plasma K_2-MK-7 and K_2-MK-4 concentration, without further improvement in endothelial function. In ApoE/LDLR$^{-/-}$ mice with developed atherosclerotic plaques, treatment with a low (0.03 mg/kg) or high (10 mg/kg) dose of K_2-MK-7 resulted in a similar degree of endothelium-dependent vasodilation improvement and increase in plasma nitrate concentration, what was not associated with changes in thrombin generation as measured by CAT. Both doses of K_2-MK-7 also reduced media thickness in the brachiocephalic artery, but did not modify atherosclerotic plaque size.

In conclusion, K_2-MK-7 improves NO-dependent endothelial function in ApoE/LDLR$^{-/-}$ mice. This study, identifies the endothelial profile of the pharmacological activity of vitamin K_2, which has not been previously described.

1. Introduction

The family of fat-soluble compounds termed vitamin K consists of several menaquinones (MKs, vitamin K_2), mostly originating from bacterial synthesis and single, plant-derived phylloquinone (PK, vitamin K_1). Various representative of MKs differ in the number of isoprenoid residues in the aliphatic side chain and include short- as well as long-chain isoprenoid-containing vitamins K_2 (from MK-4 to MK-13) [1]. Importantly, PK and various MKs, including a widely studied K_2-MK-7, seem to be converted endogenously to MK-4 in various organs, by the activity of the tissue MK-4 biosynthetic enzyme UbiA prenyltransferase domain-containing protein 1 (UBIAD1) [2,3].

Abbreviations: AA, abdominal aorta; Ach, acetylcholine; ApoE/LDLR$^{-/-}$, apolipoprotein E/low-density lipoprotein receptor-deficient mice; BCA, brachiocephalic artery; CAT, calibrated automated thrombography; EPR, electron paramagnetic resonance; FA, femoral artery; FMD, flow mediated dilatation; IVA, internal vessel area; LCA, left common carotid artery; LC-APCI-MS/MS, liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization technique; MGP, matrix Gla-protein; MKs, menaquinones; K_2-MK-4, menaquinone-4; K_2-MK-7, menaquinone-7; MRI, magnetic resonance imaging; NO, nitric oxide; PK, phylloquinone; UBIAD1, UbiA prenyltransferase domain-containing protein 1; VKDPs, vitamin K- dependent proteins; VWA, vessel wall area

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https://doi.org/10.1016/j.vph.2019.106581
Received 4 February 2019; Received in revised form 1 August 2019; Accepted 12 August 2019
Available online 14 August 2019

Table 1
Composition of experimental diets [g/kg] and treatment doses of K_2-MK-7.

	Female mice			Male mice			
	Untreated	Dose of K_2-MK-7 [mg/kg]		Untreated	Dose of K_2-MK-7 [mg/kg]		
		0.03	10		0.05	0.5	5
Corn starch [g]	533	533	533	533	533	533	533
Caseine [g]	200	200	200	200	200	200	200
Sucrose [g]	100	100	100	100	100	100	100
Soybean oil [g]	70	70	70	70	70	70	70
Cellulose powder [g]	50	50	50	50	50	50	50
Mineral mixture [g]	35	35	35	35	35	35	35
Vitamin mixture 1[a] [g]	10	–	–	–	–	–	–
Vitamin mixture 2[b] [g]	–	10	10	10	10	10	10
Choline bitartrate [g]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
t-butylohydroquinone [g]	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Vitamin MK-7 in diet [mg]	–	0.15	50.00	–	0.25	2.50	25.00
Equivalent dose of MK-7 [mg/kg b.w.]	–	0.03	10.00	–	0.05	0.50	5.00

[a] Standard vitamin mixture containing 0.75 mg/kg (equivalent to dose 0.15 mg/kg b.w./day) vitamin K_1.
[b] Vitamin mixture without vitamin K_1.

All vitamin K homologues function as cofactors for γ-glutamyl carboxylase (GGCX), an enzyme which activates proteins containing in their structure glutamate residues by their carboxylation to γ-carboxyglutamate (Gla) required for biological activity of these proteins [4,5]. The best known activity of vitamin K is targeted to the liver, where vitamin K-dependent carboxylation was originally observed in numerous clotting factors including prothrombin, factor VII, IX and X [6,7]. However, a number of biological functions beyond coagulation have been attributed to vitamin K [8]. Indeed, evidence has accumulated that vitamin K-dependent mechanisms extend far beyond coagulation and may have a role in osteoporosis, osteoarthritis, cancer, diabetes, inflammation, neurodegeneration and vascular calcification [1,9–12]. It is still not entirely clear whether all of these effects are linked to various vitamin K-dependent proteins (VKDPs) or are mediated by other mechanisms [13]. Quite surprisingly, it has also been demonstrated, that intake of vitamin K_2 (exerting mainly extrahepatic activity), but not vitamin K_1 (exerting mainly hepatic activity), reduces cardiovascular and total mortality [14], with these protective effects not able to be explained by the profile of action of vitamin K described so far.

Given the fact that endothelial function determines cardiovascular health [15–19], positive effects of the intake of vitamin K_2 on cardiovascular mortality could be linked to the vitamin K-dependent regulation of endothelial function. The vascular endothelium maintains vascular homeostasis, while endothelial dysfunction is featured by increased vascular permeability, platelet adhesion, pro-thrombotic response, leukocyte adhesion and pro-inflammatory response [15,16]. Most importantly, endothelial dysfunction, diagnosed as an impaired endothelium dependent vasodilation, predicts adverse cardiovascular events and poor long-term outcomes in patients with cardiovascular risk factors [20,21] as well as in healthy subjects [22,23].

To the best of our knowledge, the effects of vitamin K_2 on endothelial function have not been convincingly documented as yet. Therefore, the aim of the present study was to examine the effects of vitamin K_2-MK-7 on endothelial dysfunction, in apolipoprotein E/low-density lipoprotein receptor-deficient mice (ApoE/LDLR$^{-/-}$) mice at the early and late stages of disease development, in the absence and presence of atherosclerotic plaques, respectively. To assess endothelial function a 3D magnetic resonance imaging (MRI)-based method was used, that was previously demonstrated to be suitable for reliable detection of the impairment of nitric oxide (NO)-dependent vasodilation *in vivo* [24–26], as well as for the *in vivo* endothelial profiling of compounds to demonstrate their beneficial effects on endothelial function [27].

2. Materials and methods

2.1. Animals and experimental protocol

Studies were performed in ApoE/LDLR$^{-/-}$ mice bred in the Department of Human Nutrition, University of Agriculture in Krakow (Poland). ApoE/LDLR$^{-/-}$ mice represent a murine model of hypercholesterolemia and atherosclerosis, initially described by [28] and extensively characterized by us in our previous studies [27,29–34].

In the first part of the study, male mice without well-established atherosclerotic plaque and with early phase of endothelial dysfunction (8–11 weeks of age) [35], were used to examine effects of 2-, 4- and 8-week treatment with a low dose (0.05 mg/kg b.w./day) and higher doses (0.5 and 5 mg/kg b.w./day) of vitamin K_2-MK-7. Therefore, mice were randomly assigned to four experimental groups: control (untreated group) and three groups treated with K_2-MK-7 at doses of 0.05, 0.5 and 5 mg/kg b.w./day, respectively, with end-point measurements being performed at the age of 10–16 weeks.

In the second part of the study, effects of vitamin K_2-MK-7 were analyzed in female mice with advanced endothelial dysfunction and pre-established atherosclerosis (16 weeks of age) [35] treated with K_2-MK-7 for eight weeks. In this part of the study, mice were randomly assigned to three experimental groups: control (untreated group) and two groups treated with low and high doses (0.03 and 10 mg/kg b.w./day, respectively) and end-point measurements were performed at the age of 24 weeks.

The low dose of vitamin K_2-MK-7 (0.03–0.05 mg/kg b.w./day), used in the current study after adjustment for metabolic rate of mice, is compatible with the effective doses (180–360 μg) for longer chain menaquinones (MK-7, MK-8, and MK-9) recommended for humans, to provide benefits for cardiovascular health [36,37].

The high doses were choosen based on previous work demonstrating the anti-atherosclerotic effects of vitamin K_2 [38].

Vitamin K_2-MK-7 (provided by the Pharmaceutical Research Institute (Warsaw) courtesy of dr Maresz) was dissolved in soybean oil and administrated as a part of semi-synthetic AIN 93G diet (Table 1), with or without a standard vitamin mixture containing vitamin K_1.

Mice were housed in collective cages, in a room with constant environmental conditions (22–25 °C, 65–75% humidity and 12 h light/dark cycle). Animals had *ad libitum* access to daily provided diets and water. The size of a given experimental groups is reported in the legends of the corresponding graphs. All experiments were approved by the Local Ethics Committee of Jagiellonian University (Krakow, Poland) and were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (NIH

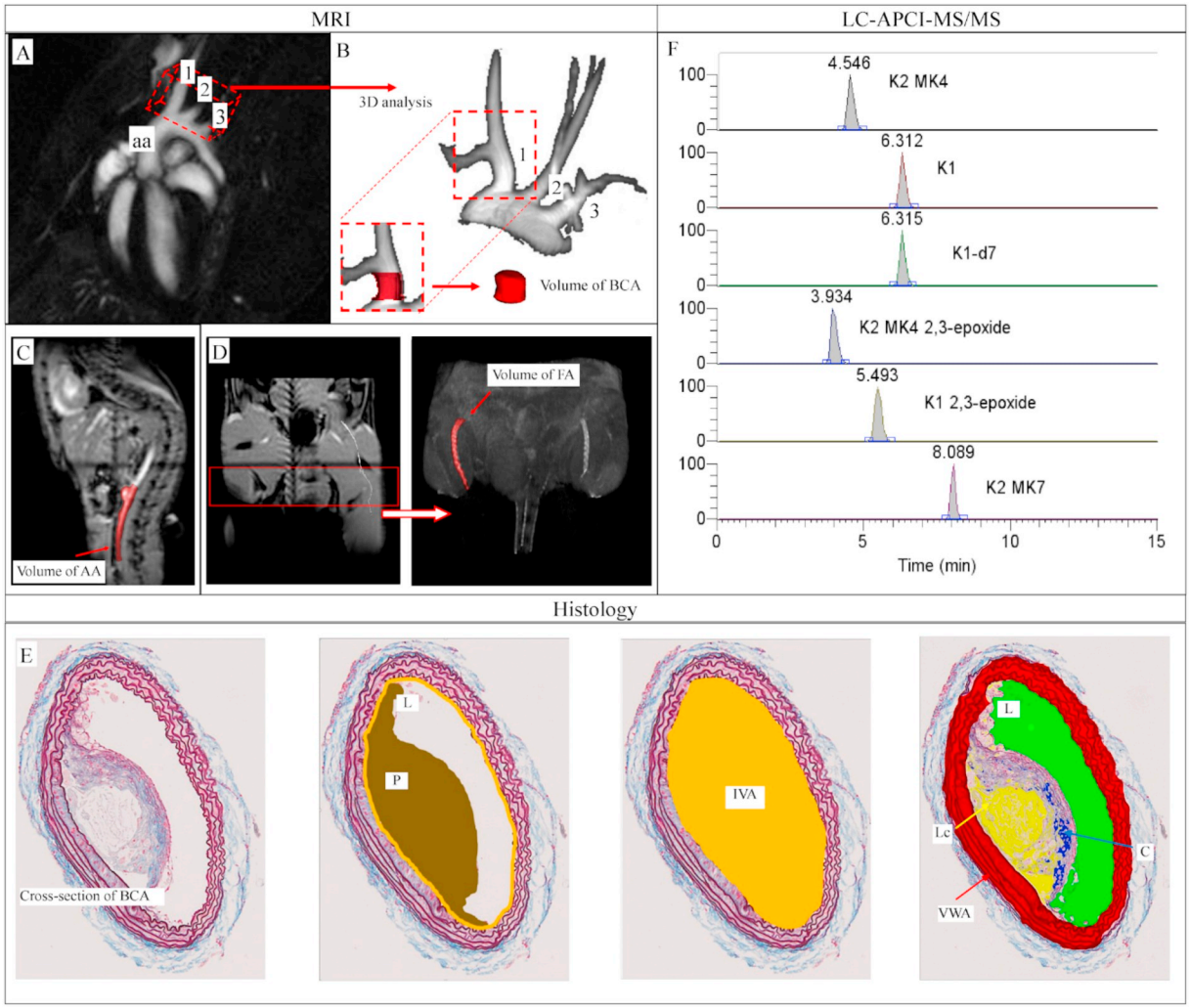

Fig. 1. Methodology of MRI-based assessment of endothelium-dependent response *in vivo* (A-D), assessment of plaque size and composition using histological analysis (E) and LC-APCI-MS/MS -based assessment of plasma concentration of vitamin K (F). A: Four-chamber view of the heart with aortic arch (aa). Image showing position of the imaging layer (red cuboid) used for 3D MRI imaging of the aa. Additionally, the following vessels are visible: (1) brachiocephalic artery (BCA), (2) left common carotid artery (LCA) and (3) subclavian artery (LSA). B: 3D image of aa acquired with the cine IntraGate™ FLASH 3D sequence. C: Sagittal view of the cross-section of mice with abdominal aorta (AA). D: Coronal view of mice with femoral artery (FA). Endothelial function assessment, expressed as changes in vessel volume, was performed in BCA, LCA, AA and FA. E: Representative images of the BCA cross-sections stained with Unna's orcein combined with Martius, Scarlet and Blue trichrom (OMSB). The areas of particular components of atherosclerotic plaque (P) including: lipid core (Lc), collagen (C), as well as artery lumen (L) and vessel wall area (VWA) were determined after Columbus-based software processing. Internal vessel area (IVA) was determined as the sum of plaque area and lumen area. F: LC-APCI-MS/MS chromatograms of the various vitamin K homologues.

publication No. 85–23, revised 1996), as well as the Guidelines for Animal Care and Treatment of the European Community.

2.2. Assessment of endothelium-dependent vasodilation in vivo by magnetic resonance imaging (MRI)

MRI experiments were performed using a 9.4 T scanner (BioSpec 94/20 USR, Bruker, Germany), located in the Department of Magnetic Resonance Imaging, Institute of Nuclear Physics, Polish Academy of Sciences in Krakow. During the experiment, mice were anesthetized using isoflurane (Aerrane, Baxter Sp. z o. o., Poland, 1.7 vol%) in an oxygen and air (1:2) mixture and imaged in the supine position. Activity of the heart, respiration and body temperature (maintained at 37 °C using circulating warm water) were monitored using a Monitoring and Gating System (SA Instruments Inc., Stony Brook, NY, USA).

Endothelial function was assessed based on vascular responses to acetylcholine (Ach) administration in the brachiocephalic artery (BCA), the left common carotid artery (LCA) (Fig. 1 A–B) and the abdominal aorta (AA) (Fig. 1C) as well as on response to an increase in flow (flow-mediated dilatation, FMD) in the femoral artery (FA) (Fig. 1D) as described previously [25–27]. Vasomotor response was examined by comparing two, time-resolved 3D images of the vessels, prior to and 25 min after intraperitoneal Ach administration (Sigma-Aldrich, Poznań Poland: 50 µl, 16.6 mg/kg), as well as prior to, and five minutes after vessel occlusion (description of the vessel occluder is provided elsewhere [26]). Time of the maximum response to Ach administration was determined experimentally in our previous study [25], while time of the vessel occlusion (ischemia) was established based on another report [39]. As reported earlier, Ach-induced response in the BCA was fully blocked by N^G-nitro L-arginine methyl ester (L-NAME), supporting the notion that this response was mediated by NO [25]. Similarly, Ach-induced response in the aorta and FMD in the FA were also fully blocked by L-NAME (unpublished results). Images were acquired using the cine IntraGate™ FLASH 3D sequence, reconstructed with the IntraGate 1.2.b.2 macro (Bruker). End-diastolic volumes of vessels were analyzed using ImageJ software 1.46r (NIH Bethesda, Maryland, USA) and scripts written in Matlab (MathWorks, Natick, MA, USA). Details of image analyses are provided in supplementary materials of our previous

work [25]. Imaging parameters included the following: repetition time (TR) - 6.4 ms, echo time (TE) - 1.4 ms, field of view (FOV) - 30x30x5 mm^3, matrix size - 256x256x30, flip angle - 30°, and number of accumulations (NA) - 15, reconstructed to seven cardiac frames. Total scan time was 10 min.

2.3. Assessment of endothelial NO production in aorta using electron paramagnetic resonance (EPR)

For *ex vivo* measurements of endothelial nitric oxide synthase (eNOS)-dependent NO production, EPR spin-trapping with diethyldithiocarbamic acid sodium salt (DETC) was used, as described previously [40], with minor modifications. Briefly, isolated aorta cleared from surrounding tissue, was opened longitudinally and preincubated with 10 μM N6-(1-Iminoethyl)-lysine, hydrochloride (L-NIL) in Krebs-HEPES buffer for 30 min at 37 °C in a well of a 48-well plate. Next, 250 μl of $Fe(DETC)_2$ colloid was added and aorta was stimulated with calcium ionophore A23187 (the final concentration: 1 μM) and subsequently, incubated for 90 min at 37 °C. Finally, the aorta was weighed and frozen in liquid nitrogen (suspended in fresh buffer) into the middle of a 400 μl column of Krebs–Hepes buffer and stored at −80 °C until measured. EPR spectra were obtained using an X-band EPR spectrometer (EMX Plus, Bruker, Germany), equipped with a rectangular resonator cavity H102. Signals were quantified by measuring the total amplitude of the NO-$Fe(DETC)_2$ after baseline correction. The quantitative results of NO production assessed by EPR, were expressed in AU/mg of tissue.

2.4. Blood sampling and biochemical analysis

After *in vivo* MRI measurements, the mice were injected intraperitoneally with 1000 IU of heparin (Sanofi-Synthelabo; Paris, France) and after 15 min, anesthetized intraperitoneally with mixture of ketamine and xylazine in doses of 100 and 10 mg/kg b.w. respectively. Blood samples were collected from the heart into test tubes containing additional anticoagulant. On the same day, 25 μl of whole blood was used for blood count analysis, using an automatic hematology analyzer ABC Vet (Horiba, Germany). The remaining blood was centrifuged at 1000 × *g* for 10 min at 4 °C and plasma was deep frozen for high-performance liquid chromatography (HPLC) measurements of nitrate (NO_3^-) and nitrite (NO_2^-) concentrations by ENO-20 NOx Analyzer, or for lipid profile and liver enzyme analysis by the biochemical analyzer (ABX Pentra 400 - Horiba Medical, Kyoto, Japan). Moreover, liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization technique (LC-APCI-MS/MS)-based assessment of vitamin K derivatives concentration was performed as described below in plasma samples obtained from blood samples collected with EDTA K_2 and a Protease Inhibitor Cocktail that were centrifuged at 1000 × *g* for 10 min at 4 °C. Thrombin generation was measured in plasma samples obtained from blood samples anticoagulated with 3.2% sodium citrate (at volume ratio 9:1).

2.5. LC-APCI-MS/MS - based assessment of plasma concentration of vitamin K

To measure the concentration of vitamin K_2-MK-7 and also other vitamin K derivatives (Vitamin K_1 – phylloquinone (PK), K_2 – menaquinone-4 (MK4)) in plasma, method based on high performance liquid chromatography-tandem mass spectrometry with an atmospheric pressure chemical ionization technique (LC-APCI-MS/MS) was developed.

An aliquot of 100 μl of plasma in amber tubes was spiked with 10 μl of internal standard – vitamin K_1-d7 (1 μg/ml in ethanol). Additional ethanol (200 μl) was added to denature the protein and briefly mixed. Subsequently, 1 ml of hexane was added and shaked vigirously for 15 min. Samples were centrifuged at 15,000 rpm, for 15 min, and the upper layer was quantitatively transferred to a new tube and evaporated under a stream of nitrogen at room temperature. The residue was dissolved with 30 μl of 2-propanol, centrifuged at 15,000 rpm for 15 min and 5 μl was injected into the column.

The HPLC analyzes were conducted with an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, US). Separations were carried out using a reversed-phase PFP analytical column (Kinetex 2,6 μm PFP, 100 Å, 100.0 × 3.0 mm, Phenomenex, Torrance, CA, US) with a mobile phase consisting of 0.1% formic acid in 2-propanol (phase A) and 0.1% formic acid in 5 mM ammonium formate (phase B) in gradient elution. The chromatogram of determined vitamin K derivatives is presented in Fig. 1F.

Mass spectrometry was performed with a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, US), equipped with an APCI electrospray ion source. All MS analyses were performed in positive ionization mode in SRM mode monitoring following ion transitions: K1: 451.4 → 187.2; K1-d7: 458.5 → 192.2; K1 2,3-epoxide: 467.4 → 161.2; MK4: 445.4 → 187.2; MK4 2,3-epoxide: 461.3 → 311.1; MK7: 649.6 → 187.2. The working parameters of the mass spectrometer were as follows: corona discharge needle voltage, 4 kV, vaporiser temperature: 325 °C, sheath gas pressure 50 Arb, ion sweep gas pressure 10 Arb, auxiliary gas pressure 30 Arb, capillary temperature 325 °C and collision pressure 1.5 mTorr with Argon as the collision gas.

2.6. Thrombin generation measurements using calibrated automated thrombography (CAT)

Thrombin generation was measured in citrate-anticoagulated mice plasma using a calibrated automated thrombography (CAT) technique, as described previously [41]. Thrombin generation was activated by mixing 21 μl of diluted plasma (1:1 with BSA5 buffer) with 7 μl of fluorogenic substrate (*Z*-Gly-Gly-Arg-AMC final concentration 417 μM) and 14 μl of trigger solution containing phospholipids, tissue factor and $CaCl_2$ (final concentration 4 μM, 1 pM and 16.6 mM, respectively). In the calibration wells, the 14 μl of reagents were replaced with calibrator. Immediately after activation, 5 μl of the mixture was pipetted on paper disks in a flat bottom 96-well polystyrene plate and covered with 40 μl of mineral oil. Fluorescent signals were measured using Fluoroskan Ascent software (Thermo Labsystems, Helsinki, Finland) and transformed into thrombin concentration as described previously [42]. The parameters analyzed included endogenous thrombin potential (ETP), peak thrombin concentration (peak) and lag time. All reagents were provided by Synapse BV, Netherlands.

2.7. Histological assessment of area and composition of atherosclerotic plaque (cross-section method)

For determination of atherosclerotic plaque area and composition, isolated BCA was dissected, fixed in 4% buffered formalin and embedded in paraffin. 5 μm-thick serial sections of BCA were collected from the proximal to the distal part of the artery. Our originally developed staining method with Unna's orcein combined with Martius, Scarlet and Blue trichrome (OMSB), was applied on every tenth section (50 μm interval between each section) for quantitative analysis of atherosclerotic plaques as described recently by us elsewhere [43]. The areas of particular components of atherosclerotic plaque as well as artery lumen and wall were determined after Columbus-based software processing (Fig. 1E) using a specially-designed algorithm [43]. The parameters analyzed include vessel wall area (VWA), internal vessel area (IVA = plaque area + lumen area), plaque area (expressed as percent of internal vessel area: plaque/IVA), lumen area (expressed as percent of internal vessel area: lumen/IVA) and areas of collagen and lipids in plaque (expressed as percent of plaque area: collagen/plaque and lipid/plaque, respectively).

2.8. Immunohistochemical detection of macrophages in atherosclerotic plaque in BCA

For the detection of macrophages in BCA, atherosclerotic plaque immunohistochemical staining was performed. In brief, after heat-induced epitope retrieval, with 10 mM sodium citrate buffer (pH 6), deparaffinised sections were blocked with 5% normal goat serum and 1% hydrogen peroxide. Macrophages were revealed by overnight incubation with monoclonal anti-Mac-3 antibody (BD Pharmingen, 550,292). After this procedure, sections were treated with biotinylated secondary antibody, followed by horseradish peroxidase-conjugated streptavidin. 3,3′-diaminobenzidine (DAB) was used for visualisation of antibody-antigen interactions. Sections were counterstained with haematoxylin. Intensity of Mac-3 staining was estimated manually considering the quantity and intensity of positive reactions. Final results are shown as a mean from two independent analyses.

2.9. Statistical analysis

Obtained data are presented as mean and standard deviation (SD) or in the case of a lack of normal distribution (Shapiro–Wilk test), as median and interquartile range (IQR). Statistical tests were performed using STATISTICA 10 (Stat Soft Inc., USA). Non-parametric (Kruskal Wallis test or Mann-Whitney *U* test) or parametric (one- and two-way analysis of variance (ANOVA) with Tukey's test, unpaired t-Student test) tests were performed. A value of $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of vitamin K_2-MK-7 treatment on nitric oxide-dependent endothelial function in young ApoE/LDLR$^{-/-}$ mice prior to the development advanced atherosclerotic plaque

As shown in Fig. 2 A–B, a 4-week treatment with a low dose (0.05 mg/kg b.w./day) of vitamin K_2 MK-7, improved FMD in femoral artery (FMD-FA, volume changes: 33.26% in mice treated with K_2 - MK-7 and 17.45% in untreated mice) as well as improved the Ach response in abdominal aorta (ACH-AA, volume changes: −1.59% in mice treated with K_2-MK-7 and -11.22% in untreated mice) in 15-week-old treated ApoE/LDLR$^{-/-}$ mice as compared with untreated ApoE/LDLR$^{-/-}$ mice. At this age, ApoE/LDLR$^{-/-}$ mice are characterized by a full-blown phenotype of endothelial dysfunction but not yet by advanced atherosclerosis plaques [34,35].

In contrast to the 4-week period of vitamin K_2-MK-7 treatment, a 2-week period of treatment with a low dose of vitamin K_2-MK-7 (0.05 mg/kg b.w./day) was not sufficient to improve endothelial function, as evidenced by the lack of change in the magnitude of vasodilation in response to increased flow in the femoral artery (FMD-FA, Fig. 2A) and in the magnitude of the endothelium-dependent response to ach in the abdominal aorta (ACH-AA, Fig. 2B). However, higher doses of vitamin K_2-MK-7 improved endothelial function after only two weeks of treatment (volume changes: 28.57% in FMD-FA, and 1.52% in ACH-AA, $p = .05$ for the dose of 0.5 mg/kg b.w./day as compared with 20.86% in FMD-FA and −7.72% in ACH-AA in untreated mice, respectively).

Interestingly, the improvement in endothelial function afforded by four weeks of treatment with higher doses of K_2-MK-7 (0.5 mg/kg b.w./day and 5 mg/kg b.w./day) was not superior as compared with that achieved by a low dose treatment with K_2-MK-7 administered for four weeks (Fig. 2 A-B), despite the fact, that three doses of vitamin K_2-MK-7 (0.05; 0.5; 5 mg/kg b.w./day) resulted in a dose-dependent increase in plasma concentration of K_2-MK-7 in number of samples for which concentration of vitamin K_2 was above limit of detection (Fig. 3A). Vitamin K_2–MK-4 plasma concentration, also increased in majority of samples in response to treatment with higher doses of vitamin K_2–MK-7 (Fig. 3B). Of note, the concentration of MK-4 2,3-epoxide (Fig. 3E) in plasma was maintained at a similar level in all groups, while vitamin K_1 (Fig. 3C) and K_1 2,3-epoxide (Fig. 3D) plasma concentration tended to decrease with increasing dose of vitamin K_2-MK-7.

As shown in Fig. 2C-D, prolongation of the treatment time to eight weeks was also not associated with a superior effect of treatment as compared with K_2-MK-7 administered at low dose (0.05 mg/kg b.w./day).

The positive effects of a the low dose of vitamin K_2 (0.05 mg/kg) improving the endothelium-dependent vasodilation measured *in vivo,* was confirmed by increased nitric oxide production measured in the same animals by EPR in aorta *ex vivo,* taken from ApoE/LDLR$^{-/-}$ mice treated with a low dose (0.05 mg/kg b.w./day) of vitamin K_2-MK-7 (Fig. 2E) as compared with untreated, age-matched ApoE/LDLR$^{-/-}$ mice.

3.2. Effects of vitamin K_2-MK-7 treatment on endothelial function in older ApoE/LDLR$^{-/-}$ mice at advanced atherosclerosis

In 24-week-old non-treated ApoE/LDLR$^{-/-}$ mice, characterized by advanced endothelial dysfunction and the presence of atherosclerotic plaques [35], injection of Ach resulted in constriction of BCA and LCA amounting to −9.13% in BCA and −28.33% in LCA. As shown in Fig. 4 A-B, 8-week treatment with a low dose of vitamin K_2-MK-7 (0.03 mg/kg b.w./day) resulted in improvement of endothelial function as evidenced by partial reversal of the Ach-induced vasoconstriction response. The change in volume of BCA (Fig. 4A) and LCA (Fig. 4B) after Ach injection was approximately 3% and −3%, respectively. Treatment of ApoE/LDLR$^{-/-}$ mice for eight weeks with a high dose (10 mg/kg b.w/day) of vitamin K_2-MK-7 also resulted in the improved endothelium-dependent vasodilation induced by Ach in LCA and BCA. The mean results for the Ach-induced response in groups treated with a low (volume changes: 3.25% and −3.92% for BCA and LCA respectively) and high (volume changes: 3.12% and 4.45% for BCA and LCA respectively) dose of vitamin K_2-MK-7 were similar. However, the improvement of endothelial function in the high dose group was more significant due to more homogenous response and lower standard errors.

Improvement of endothelium-dependent vasodilation by treatment of older ApoE/LDLR$^{-/-}$ mice for eight weeks with a low (0.03 mg/kg b.w./day) as well as high dose (10 mg/kg b.w./day) of vitamin K_2-MK-7 resulted in an increased nitrite (NO_2^-) concentration (Fig. 4C) in plasma (by about 58% and 42%, respectively). The concentration of nitrate (NO_3^-) (Fig. 4D) in plasma did not change significantly in ApoE/LDLR$^{-/-}$ mice treated with vitamin K_2-MK-7 at doses of 0.03 and 10 mg/kg b.w./day.

Eight-week treatment of ApoE/LDLR$^{-/-}$ mice with vitamin K_2-MK-7 of either dose did not affect lipid profile, liver enzymes concentration, blood cell count (Table 2) or thrombin generation (Fig. 4E–F).

3.3. Effects of treatment with vitamin K_2-MK-7 on vessel wall structure, media thickness and atherosclerotic plaque size in brachiocephalic artery in young and older ApoE/LDLR$^{-/-}$ mice

As shown in Fig. 5A, in 24-week-old ApoE/LDLR$^{-/-}$ mice treated for eight weeks with vitamin K_2-MK-7, there was a clear-cut effect on vessel wall area (VWA), the parameter representative for media layer thickness of the vascular wall as compared with 24-week-old non-treated ApoE/LDLR$^{-/-}$ mice. The effects of low (0.03 mg/kg b.w./day) and high (10 mg/kg b.w./day) dose of vitamin K_2-MK-7 were similar in magnitude (VWA decreased by approximately 5% for both low and high doses of vitamin K_2-MK-7). Noteworthy, there were some subtle differences in the results for the assessment performed separately for proximal, middle and distal parts of BCA. Effect of low dose (0.03 mg/kg b.w./day) of vitamin K_2-MK-7 was more pronounced in the middle and distal part of the vessel in ApoE/LDLR$^{-/-}$ mice, suggesting stronger effects of low dose of vitamin MK-7 on vascular wall structure in the middle part of the BCA, that is characterized by less advanced

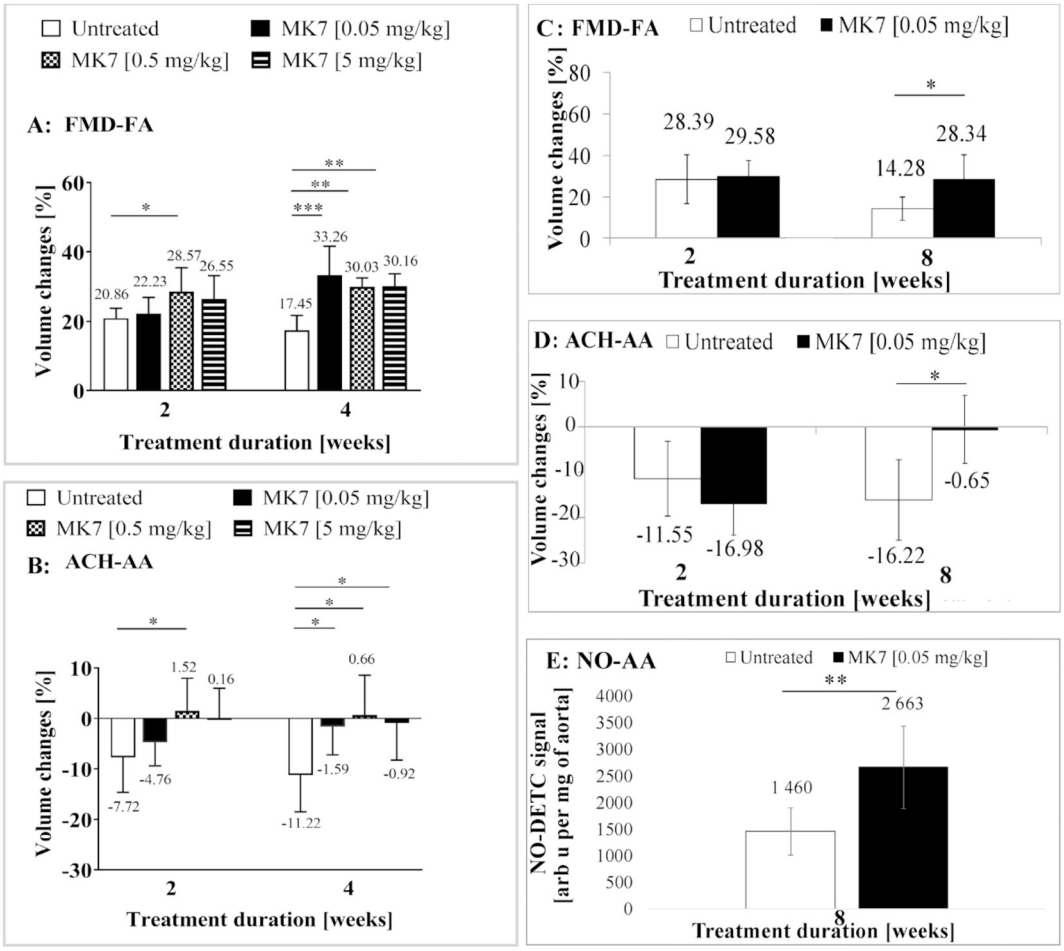

Fig. 2. Effects of treatment with vitamin K_2-MK-7, for a period of two to eight weeks, given at a dose of 0.05, 0.5, and 5 mg/kg b.w./day on endothelium-dependent vasodilation *in vivo* and on nitric oxide production in aorta *ex vivo* in young ApoE/LDLR$^{-/-}$ mice. Changes in FMD response in the femoral artery (A,C: FMD-FA) and Ach-response in the abdominal aorta (B,D: ACH-AA) measured *in vivo* by MRI and NO production (E: NO-AA) *ex vivo* in aorta measured by EPR are shown. 11-week-old ApoE/LDLR$^{-/-}$ mice were treated with vitamin K_2 - MK-7 given at a dose of 0.05 mg/kg b.w./day (n = 8, black columns), 0.5 mg/kg b.w./day (n = 5–8, columns in chessboard pattern) or 5 mg/kg b.w./day (n = 6–8, columns with horizontal lines) for two (measurements at 13 weeks of age) and four weeks (measurements at 15 weeks of age) (A,B). 8-week-old ApoE/LDLR$^{-/-}$ mice were treated with vitamin K_2-MK-7 given at dose of 0.05 mg/kg b.w./day (n = 6, black columns), for two weeks (measurements at 10 weeks of age) and eight weeks (measurements at 16 weeks of age) (C, D, E). Results were compared to untreated age-matched ApoE/LDLR$^{-/-}$ mice (n = 7–8, white columns). Statistics: A, B, C, D: 2-way ANOVA (*post hoc*: Tukey's test); E: Mann-Whitney U test, *p < .05, **p < .01, ***p < .001.

vascular pathology as compared with proximal and distal parts.

The plaque size in 24-week-old ApoE/LDLR$^{-/-}$ mice treated for eight weeks with vitamin K_2-MK-7 either at a low (0.03 mg/kg b.w./day) or high dose (10 mg/kg b.w./day) was not different as compared with non-treated 24-week-old ApoE/LDLR$^{-/-}$ mice. Internal vessel area (IVA, Fig. 5B), which is the sum of plaque and lumen areas, decreased only in the middle part of the vessel, in mice treated with a lower dose of vitamin K_2-MK-7 (data not shown), but this effect was not visible for the entire vessel. Consequently, plaque and lumen area expressed as percent of IVA (Fig. 5C–E) did not change in mice treated with either a low or high doses of vitamin K_2-MK-7.

However, despite the lack of differences between treated and untreated groups in the content of collagen in plaque (Fig. 5E), some other significant changes in atherosclerotic plaque composition were observed. In ApoE/LDLR$^{-/-}$ mice treated with low (0.03 mg/kg b.w./day) or high (0.03 mg/kg b.w./day) dose of vitamin MK-7 lipid core expressed as percent of plaque (Fig. 5F) decreased after two-month treatment either with lower or higher dose of vitamin MK-7. Specifically, changes in the content of lipids in plaque were visible in the proximal part of the BCA with least advanced plaques, but not in the distal part with most advanced plaques. The lack of effects of vitamin K_2-MK-7 on atherosclerotic plaque size was compatible with the unchanged expression of MAC in atherosclerotic plaques, suggesting similar macrophage contents of the atherosclerotic plaques in treated and non-treated 24-month-old ApoE/LDLR$^{-/-}$ mice (Intensity of MAC-3 staining: 92 ± 28 AU in untreated mice; 128 ± 26 AU in mice treated with K_2-MK-7 in low dose (0.03 mg/kg b.w./day); 139 ± 29 AU in mice treated with K_2-MK-7 in high dose (10 mg/kg b.w./day)).

Moreover, the plaque size in 16-week-old ApoE/LDLR$^{-/-}$ mice treated for eight weeks with vitamin K_2-MK-7 at a low (0.05 mg/kg b.w./day) dose (Plaque/IVA: 6.3%) was not different as compared with non-treated 16-week-old ApoE/LDLR$^{-/-}$ mice (Plaque/IVA: 3.3%). Consequently, lumen area expressed as percent of IVA did not change in mice treated with low doses of vitamin K_2-MK-7 (Lumen/IVA: 93.72% in comparison to 96.87% in non-treated 16-week-old ApoE/LDLR$^{-/-}$ mice).

4. Discussion

In the present work, taking advantage of a state-of-the-art, previously validated method to measure endothelial function *in vivo* (MRI) [25–27], we demonstrated to our knowledge for the first time, that vitamin K_2-MK-7 improved NO-dependent endothelial function in ApoE/LDLR$^{-/-}$ mice. Our results showed that vitamin K_2-MK-7

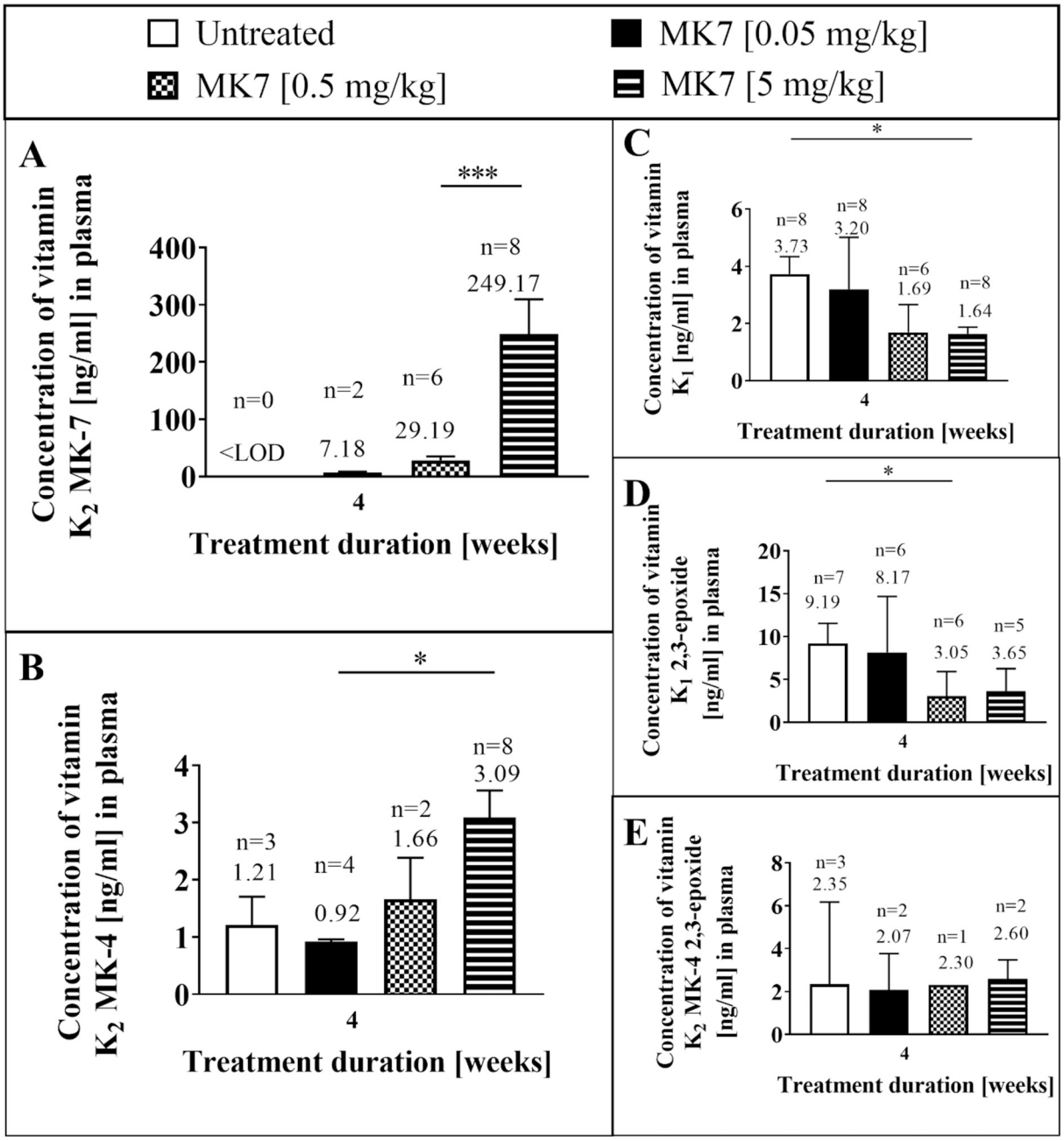

Fig. 3. Dose-dependent effects of treatment with vitamin K_2-MK-7 on plasma concentration of vitamin K_2–MK-7 and K_2–MK-4 in young ApoE/LDLR$^{-/-}$ mice. Plasma concentrations of vitamin K_2 (MK-7) (A), K_2 (MK-4) (B), K_1 (C), K_1 2,3-epoxide (D) and MK-4 2,3-epoxide (E) are shown. 11-week-old ApoE/LDLR$^{-/-}$ mice were treated for four weeks with K_2-MK-7 given at three doses (0.05 mg/kg b.w./day, the total number of samples = 8, black columns; 0.5 mg/kg b.w./day: the total number of samples = 6, chessboard pattern columns; 5 mg/kg b.w./day: the total number of samples =8; columns with horizontal lines) and compared to untreated age-matched ApoE/LDLR$^{-/-}$ mice (the total number of samples = 8, white columns). < LOD - < limit of detection, n - number of samples for which concentration of compound was above LOD (50 pg/ml). Statistics: Kruskal- Wallis test (C-D) and U-Mann Whitney test (A, B, E - Due to small number of samples, for which concentration of compound was above LOD, analysis was performed only between groups, where the number of samples was at least 4); *p < .05, **p < .01, ***p < .001.

afforded a vasoprotective effect independently whether endothelial dysfunction was treated with vitamin K_2-MK-7 prior to or concurrently with the occurrence of atherosclerotic plaques in ApoE/LDLR$^{-/-}$ mice, suggesting that the vitamin K_2-MK-7 -induced effect on endothelial function was not linked to possible anti-atherosclerotic effects of vitamin K_2 described previously [38]. Furthermore, we did not confirm any significant effects of vitamin K_2-MK-7 treatment on atherosclerotic plaque size and macrophage content. K_2-MK-7-induced improvement of endothelial function was also not linked to changes in activity of coagulation factors as evidenced by unchanged thrombin generation in plasma (CAT). Altogether, our results demonstrate that a low dose of vitamin K_2-MK-7 compatible with effective doses for K_2-MK-7, recommended for humans, to provide benefits for cardiovascular health [36,37], plays an important role in the regulation of endothelial function.

In our study, taking advantage of the state-of-the-art MRI-based method used previously to detect endothelial function *in vivo* [25–27], we showed that a low dose of vitamin K_2–MK-7 improved endothelium-dependent responses induced by ach in the aorta and induced by increased flow in the femoral aorta - responses shown previously to be

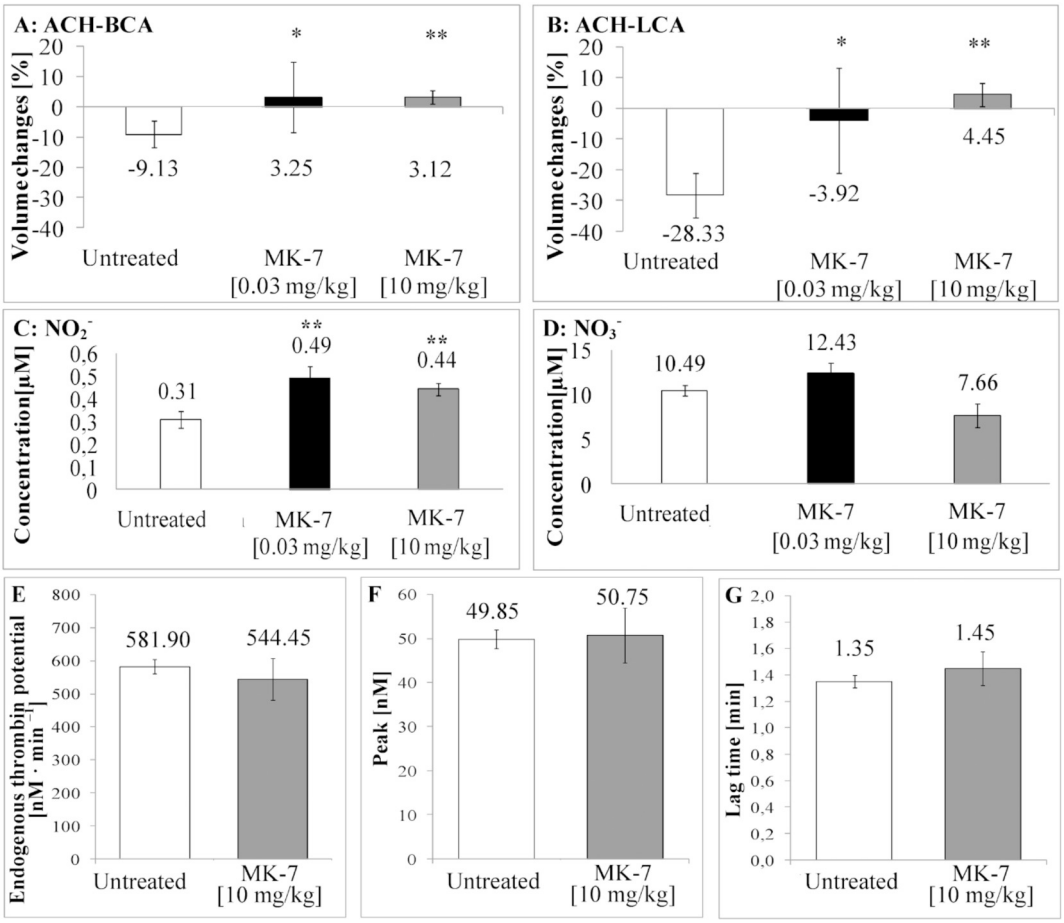

Fig. 4. Effects of treatment with low and high doses of vitamin K_2-MK-7 on endothelial function *in vivo* (A-B), nitrite (NO_2^-) and nitrate (NO_3^-) concentration in plasma (C-D) and coagulation measured by thrombin generation in plasma (E -G) in older ApoE/LDLR$^{-/-}$ mice. Changes in Ach-induced response in the brachiocephalic artery (A: ACH-BCA) and left common carotid artery (B: ACH-LCA) are shown as well as changes in NO_2^- (C) and NO_3^- (D) concentration in plasma and thrombin generation shown as endogenous thrombin potential (E), peak thrombin concentration (F: Peak) and lag time (G). 24-week-old ApoE/LDLR$^{-/-}$ mice were treated for eight weeks with vitamin K_2-MK-7 in low dose (0.03 mg/kg b.w./day: n = 8–9, black columns) or high dose (10 mg/kg b.w./day: n = 6, gray columns) and compared to untreated age-matched ApoE/LDLR$^{-/-}$ mice (n = 6–10, white columns). Statistics: A-D: one-way ANOVA; E-G: Mann-Whitney U test, $^*p < .05$, $^{**}p < .01$ *vs.* Untreated mice.

mediated by endothelial NO in mice [25,35]. The improvement of endothelial function by treatment with vitamin K_2-MK-7 was confirmed *ex vivo* by EPR measurements in the aorta using previously described methodology [40], showing increased NO production in the aorta taken from vitamin K_2-MK-7-treated ApoE/LDLR$^{-/-}$ mice as compared with non-treated animals. The improvement of endothelium dependent vasodilation by vitamin K_2-MK7 also correlated with increased nitrite plasma concentration, a reliable marker of endothelial function [44].

The treatment with a low dose of K_2–MK-7 (0.05 mg/kg b.w./day) resulted in barely detectable levels of K_2-MK-7 in plasma (detected in two mice in the group of six mice) suggesting that K_2-MK-7 in the low nanomolar range of concentration is endowed with pharmacological efficacy to improve endothelial function. We did not measure the concentration of vitamin K_2-MK-7 in tissues, and it could well be that vitamin K_2 –MK7, having relatively high bioavailability [45] and undergoing selective tissue distribution in the body, reached higher concentrations in target organs, e.g. in aorta, than in blood plasma [46]. Since K_2-MK-7 can be converted *in vivo* to K_2-MK-4 *via* UbiA prenyltransferase domain-containing protein 1 (UBIAD1) [1,47], it seems possible that the improvement in vascular function afforded by K_2-MK-7 could be due to transformation to MK-4, the latter shown to be essential for the survival of vascular endothelial cells [48]. It remains to be determined whether observed effects are induced directly by K_2-MK-7 or by K_2-MK-4 after MK-7 metabolism.

It was also showed, that vitamin K 2,3-epoxide reductase complex subunit 1-like1 (VCORC1L1), an enzyme of vitamin K cycle, is responsible for driving vitamin K-mediated intracellular antioxidation pathways [49]. Yet most likely *VCORC1L1* does not contribute to K-mediated improvement of endothelial function, as concentration of K_2–MK-4 2,3-epoxide did not changed.

This work demonstrates that vitamin K_2-MK-7 action was achieved with a relatively low dose of vitamin K_2-MK-7 associated with low nanomolar range of plasma vitamin K_2 concentration and higher doses did not result in better effects. These results suggest a saturable endothelial mechanisms of vitamin K_2-MK-7 action. Up-to-date the enzyme kinetics of vitamin K enzymes were not studied in endothelial cells, but studies using other cells or enzymes have shown, that both γ-glutamyl carboxylase [50] or vitamin-K-epoxide reductase (VKOR) [49] have low maximal velocity Vmax, in the order of pmol/h/mg of protein. UBIAD-1 enzyme,has also low Vmax in the order of pmol of protein [51]. Therefore one may speculate, that such a low dose of vitamin K_2-MK-7 was sufficient to saturate vitamin K_2-dependent mechanisms in endothelium, but further studies are needed to understand this phenomenon better.

In the present work, we did not study the mechanisms involved in K_2-MK-7–induced improvement of endothelial function. However, we confirmed that, the endothelial effects of K_2-MK-7 were independent of the coagulation system, as evidenced by unchanged thrombin

Table 2
Results of lipid profile, liver enzymes and blood count in 24-week-old ApoE/LDLR$^{-/-}$ mice treated for eight weeks with low or high dose of vitamin K_2-MK-7 in comparison to untreated age-matched ApoE/LDLR$^{-/-}$ mice.

	Untreated mice	MK-7	MK-7
		0.03 mg/kg	10 mg/kg
Lipid profile			
TC (mmol/l)	15.96 ± 4.21	19.96 ± 3.82	16.59 ± 2.89
Triglycerides (mmol/l)	1.24 ± 0.57	2.15 ± 0.46	1.87 ± 0.98
Liver enzymes			
ALT (U/l)	46.26 ± 17.97	45.20 ± 15.72	47.58 ± 19.55
AST (U/l)	108.48 ± 24.97	97.99 ± 27.89	74.40 ± 20.44
SAA (μg/ml)	35.92 ± 4.48	34.23 ± 10.42	36.63 ± 8.06
Blood count			
GRA	0.64 ± 0.13	0.73 ± 0.24	0.52 ± 0.22
GRA (%)	16.78 ± 2.89	16.00 ± 6.36	14.08 ± 3.73
Haematocrit	45.57 ± 1.26	49.99 ± 9.02	44.07 ± 7.50
HGB	12.61 ± 1.29	13.81 ± 2.53	12.09 ± 1.77
LYM	2.15 ± 0.58	3.10 ± 1.93	1.6 ± 1.25
LYM (%)	74.27 ± 4.66	75.45 ± 10.25	77.39 ± 4.50
MCH	14.85 ± 0.66	15.13 ± 0.38	14.57 ± 0.18
MCHC	27.74 ± 1.36	28.05 ± 0.39	27.22 ± 0.59
MCV	53.50 ± 1.00	54.00 ± 2.00	53.00 ± 1.00
MON	0.25 ± 0.10	0.30 ± 0.05	0.15 ± 0.20
MON (%)	8.95 ± 2.10	8.55 ± 2.00	8.53 ± 1.43
MPV	5.01 ± 0.19	5.20 ± 0.33	4.97 ± 0.19
PLT	726.50 ± 108.25	696.50 ± 154.38	727.02 ± 127.25
Erythrocytes	8.49 ± 0.37	9.37 ± 1.32	8.30 ± 1.21
RDW	12.50 ± 0.37	12.27 ± 0.39	12.13 ± 0.15
Leukocytes	3.05 ± 0.60	4.25 ± 1.61	2.23 ± 1.77

ALT - alanine aminotransferase, AST - aspartate aminotransferase, GRA - granulocytes, HGB - haemoglobin, LYM - lymphocytes, MCH - mean corpuscular haemoglobin, MCV - mean corpuscular volume, MON - monocytes, MPV - mean platelet volume, MCHC - mean corpuscular haemoglobin concentration, PLT - platelets, RDW - red (cell) distribution width, SAA - Serum amyloid A proteins, TC - total cholesterol. Statistics: one-way ANOVA (ALT, AST, GRA, GRA%, LYM %, MCH, MCHC, MON%) or Kruskal Wallis test (SAA, Haematocrit, HGB, LYM, MCV, MON, MPV, PLT, Erythrocytes, RDW, Leukocytes). *$p < .05$ *vs.* Untreated mice. Size of groups: Untreated mice (n = 9–10), MK-7 [10 mg/kg] (n = 9–12), MK-7 [0.03 mg/kg] group (n = 6–8).

generation in plasma using CAT, a reference method for the quantitative assessment of thrombin generation [42].

We also excluded a possibility that improvement of endothelial function by K_2-MK-7 in ApoE/LDLR$^{-/-}$ mice was linked to the inhibition of atherosclerotic plaque formation because vitamin K_2-MK-7 afforded vasoprotective effects independently, whether endothelial dysfunction was treated with vitamin K_2-MK-7 prior to or concurrently with the occurrence of atherosclerotic plaques in young and older ApoE/LDLR$^{-/-}$ mice.

Previously, we characterized the phenotype of endothelial dysfunction in young and old ApoE/LDLR$^{-/-}$ mice in relation to atherosclerotic plaque development [35] and showed that nearly all major features of endothelial dysfunction including impaired NO production, remained altered to an approximately similar extent, in aorta in pre-atherosclerotic and atherosclerotic phases, in 8- and 28-week-old ApoE/LDLR$^{-/-}$ mice, respectively. Thus, a comparative level of endothelial function improvement by the treatment with a low dose of vitamin K_2-MK-7 in young and older ApoE/LDLR$^{-/-}$ mice was not surprising.

The lack of anti-atherosclerotic effects of vitamin K_2-MK-7 shown here, stays in contrast with a study in rabbits demonstrating that vitamin K_2 treatment (1 to 10 mg/kg b.w./day) suppressed the progression of atherosclerotic plaques, intima-thickening, and lowered ester-cholesterol deposition in the aorta [38]. This discrepancy most likely may be explained by the fact that atherosclerotic plaques in rabbits represent an early phase of atherosclerosis responding better to anti-atherosclerotic treatment [52,53] as compared with atherosclerotic plaques in ApoE/LDLR$^{-/-}$ mice [29,34]. It was shown, that pravastatin at high (100 mg/kg), but not low dose (40 mg/kg) displayed a pronounced anti-atherosclerotic effect in BCA, in ApoE/LDLR$^{-/-}$ mice fed a control (CHOW) or an atherogenic (Low Carbohydrate High Protein, LCHP) diet [29], whereas lower dose of pravastatin was sufficient for inhibition of plaque rupture and subsequent thrombus formation in atherosclerotic rabbits with hyperlipidemia [52]. Accordingly, *v*itamin K_2 therapy may have weak anti-atherosclerotic effects that could be only seen in a mild model of atherosclerosis in rabbits, while in our work in ApoE/LDLR$^{-/-}$ meaningful anti-atherosclerotic effects of vitamin K_2 were not observed, despite clear-cut effects improving NO-dependent endothelial function. Moreover, anti-atherosclerotic effects of vitamin K_2-MK-7 were not observed also in 16-week-old ApoE/LDLR$^{-/-}$ mice despite the early type of plaque. However, area of early type of plaque in 16-week-old ApoE/LDLR$^{-/-}$ mice was too small (3–6%) to observe any relevant anti-atherosclerotic effects. Our results, showing the improvement of endothelial function and lack of strong anti-atherosclerotic effect of vitamin K_2 are perhaps not surprising given the complex multiparametric nature of endothelial dysfunction, that requires therapy targeted simultaneously to multiple features but not only to single characteristics of the phenotype of dysfunctional endothelium [35]. In fact, there are number of interventions improving NO-dependent endothelial function without strong anti-atherosclerotic effect [54–56]. On the other hand, inhibition of multiple mechanisms of endothelial dysfunction by statins or inhibitors of ACE results in pronounced anti-atherosclerotic effects [19].

In the present study, lack of effects of vitamin K_2-MK-7 on atherosclerotic plaque size was compatible with unchanged expression of MAC in atherosclerotic plaques, that was however quite a variable parameter for vessel to vessel thus with high values of errors. In contrast to the lack of the clear-cut anti-atherosclerotic effect, vitamin K_2-MK-7 significantly reduced media thickness. This average effect for the whole BCA, was similar in magnitude for low and high doses of vitamin K_2-MK-7 and for the low dose of vitamin K_2-MK-7 was the most pronounced in the proximal and middle part of the BCA, the parts of this artery which are changed by atherosclerosis to lesser extent as distal parts. The effects of vitamin K_2-MK-7 on media thickness could be linked to vitamin K_2 -dependent carboxylation of matrix Gla-protein (MGP) in smooth muscle cells, a previously well-described mechanism of vitamin K_2 [57]. Hence, a low dose of vitamin K_2-MK-7 was sufficient to improve the carboxylation status in smooth muscle cells of vascular wall in ApoE/LDLR$^{-/-}$ mice. Whether improvement of endothelial NO-dependent function was linked to improved carboxylation status in endothelium, to MGP or to other VKDPs remains to be determined.

A number of VKDPs were identified in the endothelium, including MGP, an important inhibitor of vascular calcification [58], growth arrest-specific protein-6 (Gas6) involved in endothelial survival [59-61] and osteocalcin (OC) [62]. Interestingly, in African American haemodialysis patients, higher plasma concentration of inactive MGP was associated with greater arterial stiffness as well as endothelial dysfunction [63], but this statement was merely based on the correlations between plasma dp-ucMGP concentration, pulse wave velocity (PWV) and flow-mediated vasodilation (FMD) measurements, and thus may not be entirely conclusive. At this stage, one cannot indicate which among 17 types of VKPDs identified so far play a role in the regulation of NO-dependent function. It may also be that the beneficial effects of vitamin K_2-MK-7 on smooth muscle cells [57], or anti-inflammatory effects of vitamin K_2-MK-7 [64-67], contribute to the improved endothelial function induced by vitamin K_2-MK-7. Furthermore, VKDP-independent mechanisms in endothelium or other cells should also be considered [68-70]. Clearly, further studies are required to explain the mechanism by which vitamin K_2-MK-7 improves endothelial function.

To the best of our knowledge, there are no previous studies showing effects of exogenous vitamin K_2 on endothelial dysfunction. Although, Hegarty et al. [48] reported, that vitamin K_2 maintained endothelial cell survival and demonstrated the importance of UBIAD1-mediated

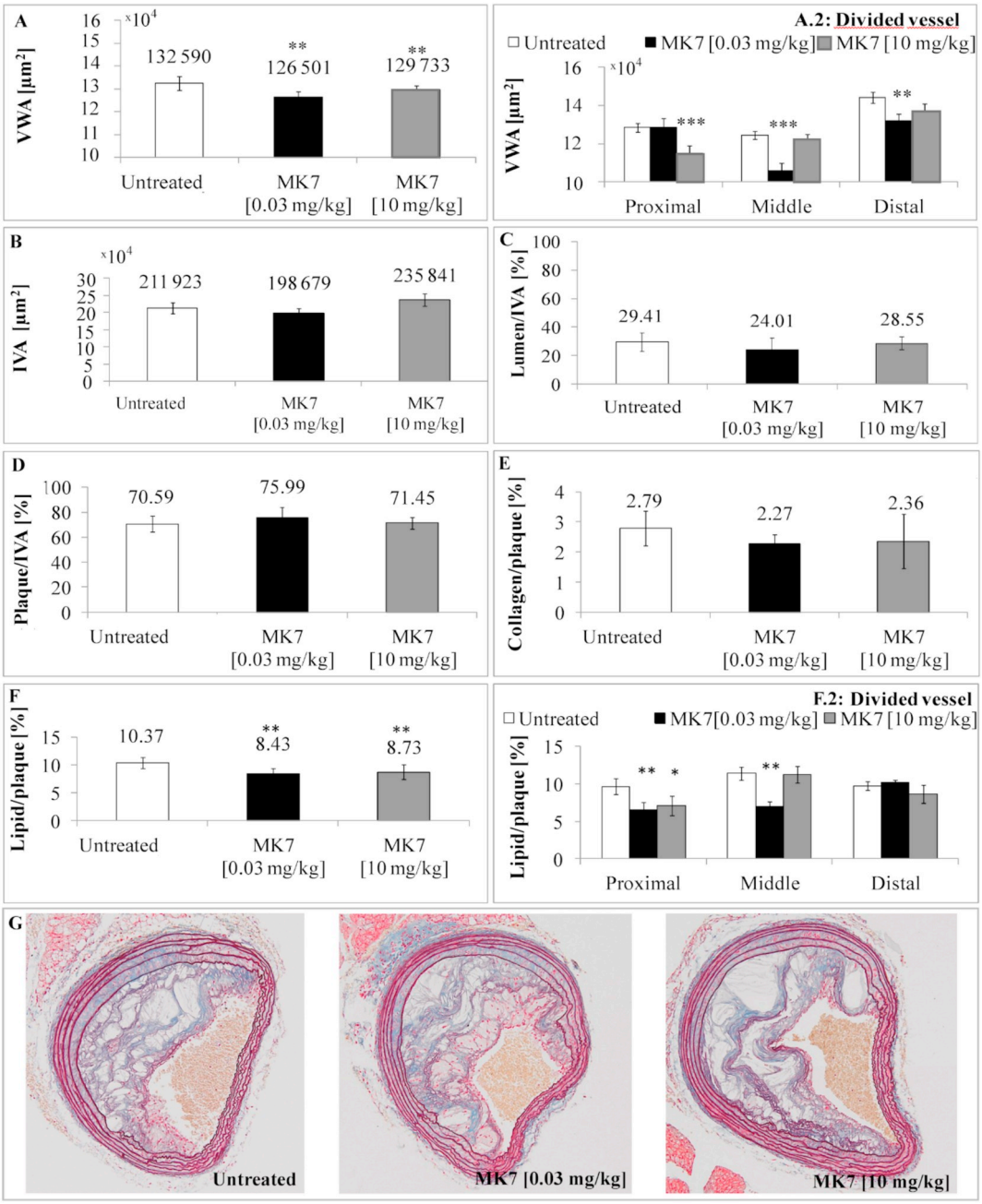

Fig. 5. Effects of treatment with low and high dose of vitamin K_2-MK-7 on vascular wall area (A), plaque size (B-D, G) and composition (E,F) in older ApoE/LDLR$^{-/-}$ mice. Vessel wall area (A: VWA) of BCA, changes in internal vessel area (B: IVA), lumen area (C: expressed as percent of internal vessel area: lumen/IVA), plaque area (D: expressed as percent of internal vessel area: plaque/IVA), areas of collagen and lipids in plaque (E: Collagen/plaque and F: Lipid/plaque, respectively) and representative images of the plaque size in the middle part of BCA (G) are shown. 24-week-old ApoE/LDLR$^{-/-}$ mice were treated for eight weeks with vitamin K_2-MK-7 in low dose (0.03 mg/kg b.w./day: $n = 7$, black columns) or high dose (10 mg/kg b.w./day: $n = 6$) as compared to untreated age-matched ApoE/LDLR$^{-/-}$ mice (n = 6, white columns). The assessment was performed for entire vessel (A-F) and for divided vessel (A.2, F.2) in proximal, middle and distal parts. Statistics: Kruskal Wallis test; $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ *vs.* Untreated mice.

vitamin K_2 synthesis in endothelium, this study related to the role of endogenous vitamin K_2. Whether exogenous vitamin K_2–MK7 used here improves NO-dependent endothelial function *via* UBIAD1-mediated endogenous vitamin K_2-MK4 synthesis or directly *via* exogenous K_2–MK7 –mediated mechanism remains to be determined.

This work has revealed that a low dose of vitamin K_2-MK-7 compatible with effective vasoprotective doses (180–360 µg) in humans [36,37,71] improved endothelial function. The data implies that endothelial effects of vitamin K_2-MK-7 discovered here could have contributed to the beneficial effects of vitamin K_2 on vascular health described previously [14,72,73]. Furthermore, endothelial dysfunction may also be involved in vitamin K insufficiency–associated cardiovascular mortality documented in PREVEND study [67]. However, in humans, there is still a lack of direct positive results of vitamin K on endothelial function.

In 244 healthy postmenopausal women, in a 3 year randomized intervention trial-supplementation with 180 µg MK-7, it was demonstrated to improve arterial stiffness, but effects on endothelial function were not detected [36]. In this work, endothelial function was not measured based on functional tests (flow-mediated dilatation, FMD), but only based on endothelial biomarkers (*e.g.* VCAM-1, selectin E), that are not particularly sensitive for detecting changes in endothelial

function [17]. In particular, this may have been the case if initially, endothelial function was not profoundly impaired, as was the case for patients in this trial. In another trial, in aged patients with a history of vascular disease, 6 months of daily oral intake of 100 μg vitamin K_2 (MK-7 subtype) did not improve endothelial function measured as FMD. In this trial, however, improvement of pulse wave velocity was also non-significant, suggesting that therapy was not efficient in terms of improving vascular function at all, perhaps due to the dose being too low [74].

In our study, we provided evidence that vitamin K_2-MK-7 treatment improved endothelial dysfunction in ApoE/LDLR$^{-/-}$ mice, a genetically-driven model of severe hypercholesterolemia, that represents a distinct endothelial and vascular pathology as compared with aging or menopausal women, studied in human trials showing negative results of K_2-MK-7 treatment on endothelial function. Hence, possible disease-specific effects of vitamin K_2-MK-7 treatment on endothelial function should be also taken into account.

Our study did not provide information, whether inclusion of vitamin K_1 could alter the obtained results. Epidemiological evidence suggests that intake of vitamin K_2 (exerting mainly extrahepatic activity), but not the intake of vitamin K_1 (exerting mainly hepatic activity) reduce cardiovascular and total mortality [14], so one can predict that the inclusion of vitamin K_1 into animals diet (0.15 mg/kg b.w./day) have not altered effects of vitamin K_2 described here. On the other hand, previous studies demonstrated effects of vitamin K_1 on vascular wall [75-77], and it cannot be excluded that endogenous vitamin K_2 may potentially be synthetized in endothelium from vitamin K_1 as in other cells [2]. Thus, effects of vitamin K_1 on endothelial function should be explored in the further studies.

There are some limitations of this study that need to be underlined. In the first part of this study, only male mice were used, while in the second part, assessment of endothelial function was performed in female mice. Secondly, in these two parts of our study endothelial function assessment was performed in different vessels. Despite this limitations, results showing that vitamin K_2-MK-7 had significant effects on endothelial dysfunction in both genders used in this study, in mice at various age and the effects was seen in various vascular beds, underlines that the effect of vitamin K_2-MK-7 improving endothelial function in murine model of atherosclerosis is robust and does not seem to be sex- or vascular bed-specific. Nevertheless, further studies finally excluding a possible sex-specific effects of vitamin K_2-MK-7 on vascular function in particular type of vessels are warranted.

5. Conclusions

In conclusion, even though the history of vitamin K research is quite long, from its discovery in the 1930s [78], followed by the identification of its molecular mechanisms of action in 1970s [6,7] and the recent discovery of vitamin K-dependent, MGP-dependent regulation of vascular calcification [57], to the best of our knowledge, the effects of vitamin K_2 on endothelial function have been described here for the first time and require further studies to delineate the mechanism involved. Given the fact that the endothelium is involved in most if not all disease states, either as a primary determinant of pathophysiology or as a victim of collateral damage [19,79], the finding of the beneficial effect of vitamin K_2-MK-7 on endothelial function opens novel perspectives for pharmacology of vitamin K_2 as vasoprotective agents in various diseases associated with endothelial dysfunction.

Acknowledgement

This work was supported by Polish National Science Centre grants: OPUS Grant No. 2018/29/B/NZ7/01684, PRELUDIUM Grant No. 2016/23/N/NZ5/00595 and partially by Kaydence Pharma. The authors would like to thank Agnieszka Zakrzewska for expert technical assistance and with measurements of nitrite and nitrate concentration in plasma. We would like to thank Leon Schurgers (CARIM School for Cardiovascular Diseases, Maastricht University, the Netherlands) for discussions. We would also like to thank to Dr. Bas de Laat from Synapse BV for methodological support in CAT and providing CAT reagents.

Declaration of Competing Interest

None.

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