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RESEARCH PAPER

Low phylloquinone intake deteriorates endothelial function in normolipidemic and dyslipidaemic mice

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Abstract

While the plasma phylloquinone (PK) concentration is inversely correlated with cardiovascular risk, the involvement of PK in regulating endothelial function has not been directly investigated. Therefore, in this study we assessed the effects of short-term treatment with PK-deficient diets (5–10 weeks) on endothelial function in normolipidemic 14-week-old male C57BL/6JCmd mice and age-matched dyslipidaemic male E3L.CETP mice. Our results show that in normolipidemic mice dietary PK deficiency was associated with a marked reduction of PK levels in the plasma and liver (liquid chromatography-mass spectrometry measurements) and with impaired endothelium-dependent vasodilation assessed *in vivo* by magnetic resonance imaging (MRI). Dietary PK deficiency-induced endothelial dysfunction was fully reversed by PK supplementation. In dyslipidaemic E3L.CETP mice, dietary PK deficiency exacerbated preexisting endothelial dysfunction. Furthermore, dietary PK deficiency decreased menaquinone-4 (MK-4) levels in the aorta but did not affect blood coagulation (calibrated automated thrombography), microbiota composition (culturing and next-generation sequencing), and gut menaquinone production. In conclusion, our study demonstrated for the first time that sufficient dietary PK intake supports endothelial function in normolipidemic and dyslipidaemic mice indicating nutritional significance of dietary PK in the maintenance of endothelial function in humans.

Keywords: Phylloquinone; Endothelial function; Cardiovascular risk; Dietary PK deficiency; MRI.

1. Introduction

The naturally occurring fat-soluble vitamin K family comprises phylloquinone (PK) and menaquinones (MKs; MK-4 through MK-14) [1,2]. The predominant source of PK for humans is diet, especially green vegetables (e.g. broccoli, kale) while MKs are found in meat (e.g. bovine and pork livers), fermented milk- and soybean-based products such as cheese and natto, or can be produced endogenously by gut microbiota [2,3]. Dietary vitamin K is absorbed in the small intestine, bound in the oil core of triglyceride-rich chylomicrons, and then transported in residual chylomicrons to the liver [4–7]. Vitamin K is partially stored in the liver (mainly PK) and partially transported to other tissues by the blood [5]. Very low-density lipoproteins (VLDLs), intermediate-density lipoproteins and low-density lipoproteins (LDLs) are also partially responsible for vitamin K transport [4,8–10].

The initially defined mechanism of vitamin K activity referred to vitamin K-dependent (VKD) carboxylation of coagulation factors

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and anticoagulant proteins C, S, and Z in the liver [11,12]. More recently, the importance of vitamin K, in particular of MKs, in regulating extrahepatic processes by canonical or noncanonical mechanisms was demonstrated, including soft tissue and vascular calcification, bone formation, angiogenesis, inflammation, myocardial remodeling, cell proliferation, and platelet activation [12–14].

Interestingly, number of lately published reports indicated that PK has beneficial cardiovascular activity, contrasting with the well-known conclusions of the Rotterdam epidemiological study postulating that intake of MKs but not PK was associated with reduced cardiovascular and total mortality [15]. For example, in a prospective cohort analysis conducted in over 7 thousand participants, high dietary PK intake diminished the incidence of cardiovascular events and overall mortality in elderly patients with cardiovascular disease (CVD) risk, while the effects for MKs were not observed [16]. In the Danish Diet, Cancer and Health Study enrolling over 53 thousand participants, the risk of atherosclerotic CVD (ASCVD) was inversely associated with high dietary PK intake in a similar way to high MK intake [17]. Furthermore, a high intake of PK-rich diets was associated with a reduced incidence of aortic stenosis and its clinical complications [18].

These prospective clinical trials are consistent with previous findings demonstrating that low plasma PK levels and low PK intake were associated with higher cardiovascular risk [19] and vascular calcification progression in patients treated for hypertension [20]. However, the mechanisms involved have not been elucidated. Most likely these mechanisms extend beyond effects of PK on the formation of new calcifying lesions within the aorta and coronary arteries characterized previously [21].

As endothelial function has diagnostic, prognostic and therapeutic significance in CVD [22,23], in this study we directly examined the role of PK in regulating endothelial function in normolipidemic PK-deficient mice using the magnetic resonance imaging (MRI)-based methodology to characterize endothelial function *in vivo* [24,25]. We also sought to explore whether the effect of PK in endothelium was driven by direct or indirect mechanisms involving its vascular conversion to MK-4 and alterations in gut microbiota composition, a key source of endogenous MKs. Finally, as PK is primarily transported by TG-rich lipoproteins, and PK metabolism might be altered in pathological states [26] with disturbed lipid homeostasis, we also directly examined the role of dietary PK in regulating endothelial function in the humanized dyslipidemia murine model represented by E3L.CETP mice [27–30].

2. Methods

2.1. Animals and experimental design

Male C57BL/6JCmd mice aged 12–14 weeks (N=136) were obtained from the Mossakowski Medical Research Centre of the Polish Academy of Sciences (Warszawa, Poland). Age-matched dyslipidaemic male APOE*3-Leiden.huCETP double transgenic mice (E3L.CETP; N=60) were provided by The Netherlands Organization of Applied Scientific Research, Metabolic Health Research (Leiden, Netherlands) [27–30]. All mice were kept in traditional colony cages without limited coprophagy in a room with a light/dark cycle and controlled temperature and humidity and received food and water *ad libitum*. Environmental enrichment in the form of plastic huts, gnawing sticks, hide tubes, and nesting material was provided to enhance the animal's welfare. Mice were acclimatized to the standard rodent diet (1324 TPF; Altromin Spezialfutter GmbH and Co. KG, Lage, Germany) until the commencement of experimental procedures.

A control rodent diet was prepared to provide a PK-sufficient diet containing PK at 0.75–2.00 mg/kg diet [31–34] and PK-deficient diet was designed based on PK content measured in diet ingredients. Since the daily intake of MKs (i.e. MK-4 and MK-7) in the classical diet fed to experimental animals has not yet been reported, the concentrations of MK-4 and MK-7 were measured in addition to PK in the main ingredients of the AIN-93M formula prepared according to Reeves *et al.* [33]. MK-4, MK-7 and PK were measured using liquid chromatography coupled to tandem mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS) [35,36]. The main vitamin K homologue in the AIN-93M diet ingredients was PK, while MK-4 and MK-7 concentrations were much lower than PK or undetectable (Table 1). Since the highest PK concentration was found in soybean-derived products, sunflower oil and casein (Table 1), the standard AIN-93M diet was modified, with soybean oil replaced with corn oil, and casein content decreased (Table 2). The animal dietary intake of essential nutrients was preserved by partially replacing corn starch with rice flakes (Table 2).

Table 1
Vitamin K concentrations in selected diet ingredients

Ingredient*	PK (mg/kg)	MK-4 (mg/kg)	MK-7 (mg/kg)
α-cellulose	ND	ND	ND
Soy protein	0.035	ND	0.248
Casein	0.002	0.003	ND
Rice flakes	ND	ND	ND
Soy flakes	0.683	ND	0.022
Cornstarch	ND	ND	ND
Corn oil	0.443	ND	ND
Sunflower oil	18.811	ND	ND
Soybean oil	10.340	ND	ND

* PK, phylloquinone; MK-4, menaquinone-4; and MK-7, menaquinone-7 concentrations are expressed as mg per kg of the particular ingredient. Key: ND, not detectable (levels below 0.0005 mg/kg for PK and MK-4 and 0.0025 mg/kg for MK-7).

The AIN-93M formulas were supplemented with PK (V3501; Sigma-Aldrich, Poznan, Poland) to obtain PK-sufficient diets based on corn starch or rice flakes, abbreviated as AIN-93M (+PK) and AIN-93M-RICE (+PK), respectively. Both PK-sufficient diets contained PK at ~0.75 mg/kg (measured diet concentration amount of 0.46±0.02 mg/kg, resulting in an intake of ~91.2 μg/kg body weight (BW) per day).

Not adding PK to the modified AIN-93M-RICE formula resulted in a PK-deficient diet abbreviated to AIN-93M-RICE (−PK). The deficient PK level of the AIN-93M-RICE (−PK) diet was confirmed after its preparation by quantifying PK levels at the beginning and end of the experiment using LC-APCI-MS/MS [35,36]. Since the PK levels in the AIN-93M-RICE (−PK) diet were below the limit of quantification (<0.0005 mg/kg), it was used to induce dietary PK deficiency in the studied mice. All diets were prepared by Zoolab (Krakow, Poland).

The effects of a PK-deficient diet on vascular function were assessed in normolipidemic male C57BL/6JCmd mice (n=64), allocated to the following four experimental groups shown in Figure 1A for a 10-week-long treatment regimen started at the age of 14 weeks:

- The AIN-93M (+PK) group fed the control AIN-93M diet supplemented with a sufficient amount of PK (n=16 divided into different measurements, with n=8 per measurement);
- The AIN-93M-RICE (+PK) group fed the PK-deficient AIN-93M-RICE diet with 50% rice flakes and supplemented with a sufficient amount of PK (n=16 divided into different measurements, with n=8 per measurement);

Table 2
Composition of the phylloquinone-sufficient and phylloquinone-deficient diets

Ingredient*	PK-sufficient diet		PK-deficient diet
	AIN-93M (+PK)	AIN-93M-RICE (+PK)	AIN-93M-RICE (-PK)
Cornstarch (g/kg)	465.692	20.692	20.692
Rice flakes (g/kg)	-	500.000	500.000
Casein (>85% proteins) (g/kg)	140.000	96.000	96.000
Dextrinized cornstarch (g/kg)	155.000	155.000	155.000
Sucrose(g/kg)	100.000	100.000	100.000
Corn oil (g/kg)	40.000	40.000	40.000
α-cellulose (g/kg)	50.000	39.000	39.000
Mineral mix (AIN-93M-MX) (g/kg)†	35.000	35.000	35.000
Vitamin mix (AIN-93-VX)‡ without PK (g/kg)	-	-	10.000
Vitamin mix (AIN-93-VX)‡ with PK (75 mg/kg mix) (g/kg)	10.000	10.000	-
L-cystine (g/kg)	1.800	1.800	1.800
Choline bitartrate (g/kg)	2.500	2.500	2.500
Tert-butylhydroquinone (TBHQ) (g/kg)	0.008	0.008	0.008

* The composition of selected diets is expressed as g of a specified ingredient per kg of each diet.
† Mineral mix AIN-93M-MX (reference number: MM00023; Zoolab).
‡ Vitamin mix AIN-93-VX (reference number: V00025-K; Zoolab).Abbreviation: PK, phylloquinone.

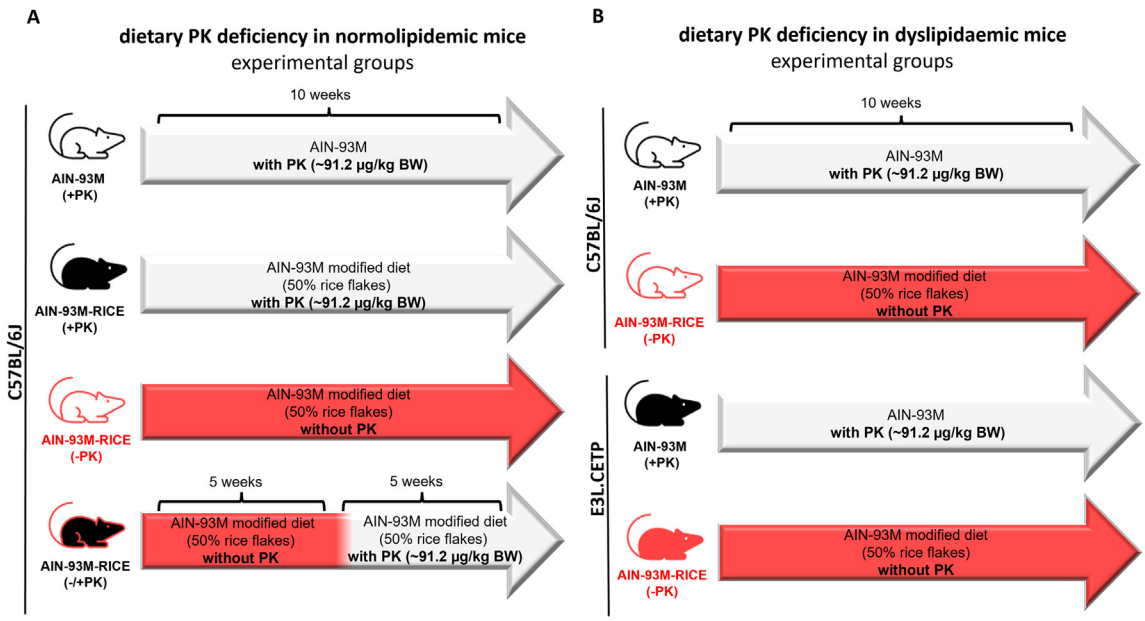

Fig. 1. Graphical description of the designed experimental groups and dietary details.

- The AIN-93M-RICE (−PK) group fed the PK-deficient AIN-93M-RICE diet with 50% rice flakes without PK supplementation (n=16 divided into different measurements, with n=8 per measurement);
- The AIN-93M-RICE (−/+PK) group fed the PK-deficient AIN-93M-RICE diet with 50% rice flakes without PK supplementation for the first 5 weeks and then supplemented with a sufficient amount of PK for the last 5 weeks (n=16 divided into different measurements, with n=8 per measurement).

Notably, the experimental design comprised two control groups, AIN-93M (+PK) and AIN-93M-RICE (+PK), to ensure that the replacement of corn starch in AIN-93M (+PK) with 50% of rice flakes in AIN-93M-RICE (+PK) did not affect the measured end-points and that the effects of dietary PK-deficiency were related directly to PK deficiency, but not to a rice flake-based diet.

Fourteen-week-old dyslipidaemic male E3L.CETP mice (n=60) were used to assess whether altered PK intake affects dyslipidemia-induced endothelial dysfunction. E3L.CETP mice represent a unique murine model of humanized dyslipidemia with a human-like lipoprotein profile and metabolism and mild hypercholesterolemia and hypertriglyceridemia [27–30]. Despite progressing dyslipidemia, E3L.CETP mice do not develop atherosclerotic plaques unless the high-fat diet is applied [29]. Obtained results were compared to age-matched normolipidemic male C57BL/6JCmd mice (n=72) treated with the same diets for 10 weeks (Fig. 1B):

- The C57BL/6J AIN-93M (+PK) group fed the control AIN-93M diet supplemented with a sufficient amount of PK (n=36 divided into different measurements, with n=6-8 per measurement);
- The C57BL/6J AIN-93M-RICE (−PK) group fed the PK-deficient AIN-93M-RICE diet without PK supplementation (n=36 divided into different measurements, with n=6–8 per measurement);
- The E3L.CETP AIN-93M (+PK) group of dyslipidaemic mice fed the control AIN-93M diet supplemented with a sufficient amount of PK (n=30 divided into different measurements, with n=6–8 per measurement);
- The E3L.CETP AIN-93M-RICE (−PK) group of dyslipidaemic mice fed the PK-deficient AIN-93M-RICE diet without PK supplementation (n=30 divided into different measurements, with n=6–8 per measurement).

The normolipidemic and dyslipidaemic mice were euthanized at the end of the experimental period using an intraperitoneal (IP) injection of ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW), then blood was collected from the right ventricle using 10% dipotassium ethylenediaminetetraacetic acid (K_2EDTA) or 3.8% sodium citrate as anticoagulants. Plasma was isolated by centrifuging the blood samples at 3000 rpm for 12 min at 4°C and then stored at −80°C until required. Next, the aorta was excised, cleaned of adherent and perivascular adipose tissue, and prepared for measurement. Part of the aorta was fixed in a 4% buffered formalin solution without cleaning for immunohistochemical analysis. The liver was also isolated, washed in ice-cold phosphate buffer saline, divided into lobes, and immediately frozen at −80°C until required.

Fecal samples were collected from each cage for vitamin K analysis within 6 h of changing the cage bedding (n=6 per experimental group collected in three replicates) and stored at −80°C until required.

During the MRI-based *in vivo* assessment of vascular function, a few mice did not survive the long-term anesthesia, and their results were excluded from further analysis (n=3 for C57BL/6JCmd and n=1 for E3L.CETP).

All animal procedures were approved by the II Local Ethical Committee on Animal Testing of the Institute of Pharmacology at the Polish Academy of Sciences (Krakow, Poland; permit nos. 271/2020 and 180/2021) and performed according to the guidelines of the European Parliamentary Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Measurement of blood coagulation, biochemical, and hematological parameters

The effect of PK-deficient diets on blood coagulation was assessed based on the plasma concentration of the thrombin-antithrombin (TAT) complex quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (EMT1020-1; AssayPro, St. Charles, MO, USA) according to the manufacturer's instructions. Thrombin generation was measured using calibrated automated thrombography (CAT) as previously described [37,38] Further details are provided in the Supplementary Materials.

Plasma triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were biochemically analyzed using a Pentra 400 analyzer (Horiba Medical, Kyoto, Japan). The non-HDL-C level was calculated by subtracting the HDL-C level from the TC level.

The serum amyloid A (SAA) concentration was determined in mouse plasma samples with a commercially available ELISA kit (ab157723; Abcam, Cambridge, UK) according to the manufacturer's instructions.

Blood counts were performed in whole murine blood samples using the scil Vet abc instrument (Horiba Medical). Hematological analysis included the following parameters: red blood cells, hemoglobin concentration, hematocrit concentration, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelets, and white blood cells.

2.3. In vivo assessment of endothelial function by magnetic resonance imaging

Vascular responses were assessed *in vivo* using MRI according to previously published protocols [38–42]. Briefly, MRIs were performed on mice with a 9.4T scanner (BioSpec 94/20 USR; Bruker BioSpin GmbH, Ettlingen, Germany) under isoflurane anesthesia with constant monitoring of body temperature, heart activity, and respiration. The endothelium-dependent vascular response to acetylcholine administration (Ach; IP at 16.6 mg/kg BW) and the endothelium-independent vascular response to sodium nitroprusside (SNP; intravenous at 1 mg/kg BW) were assessed in the thoracic aorta (ThA) and abdominal aorta (AbA). Flow-mediated vasodilation (FMD) was assessed in the femoral artery (FA) after a short-term vessel occlusion (5 min). The detailed imaging parameters and other details of this methodology are described in our previous studies [24,25] and the Supplementary Materials.

2.4. Measurement of nitric oxide systemic bioavailability and production in aorta

Systemic NO bioavailability was evaluated based on the plasma concentrations of NO metabolites, such as nitrite (NO_2^-) and nitrate (NO_3^-), using the liquid chromatography-based ENO-20 NOx Analyzer (Eicom, Kyoto, Japan) as previously described [38,43]. Aortic NO production was quantified using NO spin trapping with electron paramagnetic resonance (EPR) as previously described [38]. Further details are provided in the Supplementary Materials.

2.5. Prostacyclin measurement

Prostacyclin (PGI_2) biosynthesis was assessed based on the plasma concentration of its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), which was quantified using a commercially available ELISA kit (ADI-900-004; Enzo Life Sciences, Exeter, UK) according to the manufacturer's instructions.

2.6. Hydrogen peroxide measurements

Production of hydrogen peroxide (H_2O_2) in aorta was measured using Amplex Red kit (A22188; Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit, Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Further details are provided in the Supplementary Materials.

2.7. Immunohistochemical analysis of aorta

Aorta tissue was fixed in formalin, paraffin-embedded, and cut into 5 μm slices. The aorta tissue slides were deparaffinized, treated with a citrate buffer-based heat-induced antigen retrieval, and incubated with a blocking buffer solution containing 2% dry milk (Gostyn, Poland) and 5% of normal goat serum (Jackson ImmunoResearch, Cambridgeshire, UK) according to a standard protocol. The aorta cross-sections were immunohistochemically stained using following primary antibodies: gamma-glutamyl carboxylase (GGCX, 16209-1-AP; Proteintech, Manchester, UK), UbiA prenyltransferase domain-containing protein 1 (UBIAD1, ab191691; Ab-

cam), endothelial nitric oxide synthase (NOS-3, 610296; BD Biosciences) and its phosphorylated form (p-NOS-3, 9571; Cell Signaling Technology), von Willebrand factor (vWF, ab174290; Abcam), intercellular adhesion molecule 1 (ICAM-1, 14-0542-82; Thermo Fisher Scientific, Waltham, MA, USA), and vascular cell adhesion molecule 1 (VCAM-1, MA5-31965; Thermo Fisher Scientific). Sections were then incubated with appropriate secondary biotinylated antibodies. Color development was achieved using the ABC-HRP kit (PK-6100; Vector Laboratories, Burlingame, CA, USA) followed by incubation with 3,3′-Diaminobenzidine (DAB). The immunostained cross-sections were photographed at 100× magnification using a BX51 microscope (Olympus, Tokyo, Japan). Immunopositive pixels were quantified as previously described [38,41].

2.8. Vitamin K quantification in plasma, aorta, liver, and feces

The concentrations of PK, PK epoxide, MK-4, MK-4 epoxide, MK-5, MK-6, MK-7, MK-9, and coenzyme Q10 (CoQ10) were determined in the plasma, freshly frozen aorta, incubated aorta, liver, and feces and samples of the diets using LC-APCI-MS/MS as previously described [35,36] but with slight modification. The LC-APCI-MS/MS system comprised an Ultimate 3000 UHPLC (Dionex, Sunnyvale, CA, USA) liquid chromatograph and TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific). The best chromatographic analyte separation was achieved on a reversed-phase pentafluorophenyl analytical column (Kinetex 2.6 μm PFP, 100 Å, 100.0×3.0 mm; Phenomenex, Torrance, CA, USA) using 0.1% formic acid in 2-propanol and 0.1% formic acid in 5 mM ammonium formate as mobile phases. Detailed descriptions of the applied chromatographic method and mass spectrometry operation parameters can be found in our previous studies [35,36] and in the Supplementary Materials.

2.9. Assessment of phylloquinone uptake and its intravascular conversion to menaquinone-4

The whole aorta was isolated, cleaned of fat and adherent tissues, and incubated in minimum essential medium supplemented with 20% fetal bovine serum and 3 μM PK. After a 24-h incubation, the aorta was drained, and the tissue was weighed, immersed in 500 μL ethanol, and immediately frozen in liquid nitrogen. The samples were stored at −80°C until LC-APCI-MS/MS analysis.

2.10. Gut microbiota composition analysis

During necropsy, feces were collected from mice (n=6 per experimental group), immediately weighed, and divided into two equal parts. One part was used for quantitative microbiological cultures using classical methods. The other part was used for next-generation sequencing (NGS) analysis. Further details are provided in the Supplementary Materials.

2.11. Statistical analysis

Data are presented as the mean with 95% confidence interval (CI) and plotted using GraphPad Prism 8.2.1 software (GraphPad Software Inc., La Jolla, CA, USA). All quantitative data were statistically assessed for normality using the Shapiro–Wilk test and variance homogeneity using Levene's test. Data were compared between groups using suitable parametric (t-test or analysis of variance with Tukey's post-hoc tests) or nonparametric (Mann–Whitney U and Kruskal–Wallis tests) tests in Statistica 13.1 software (Statistica, Tulsa, OK, USA). The different levels of statistical significance are indicated on a three-cutoff p-value scale: *, $P \leq .05$; **, $P < .01$; ***, $P < .001$. The experimental mouse groups

Table 3
Body weight, selected organ weights, and hematological parameters

Parameter	Experimental groups*			
	AIN-93M (+PK)	AIN-93M-RICE (+PK)	AIN-93M-RICE (−PK)	AIN-93M-RICE (−/+PK)
Body and organ weights				
Body weight (g)	32.0 (31.3, 32.7)	31.6 (30.7, 32.6)	32.4 (31.2, 33.6)	29.7 (28.2, 31.2)†
Liver (% BW)	4.40 (4.18, 4.62)	4.74 (4.16, 5.33)	4.85 (4.50, 5.21)	4.14 (3.30, 4.98)
Lungs (% BW)	0.73 (0.64, 0.82)	0.73 (0.68, 0.79)	0.69 (0.61, 0.77)	0.77 (0.68, 0.86)
Kidneys (% BW)	1.41 (1.37, 1.45)	1.44 (1.32, 1.56)	1.39 (1.32, 1.47)	1.37 (1.32, 1.42)
Spleen (% BW)	0.36 (0.32, 0.40)	0.39 (0.31, 0.46)	0.34 (0.29, 0.39)	0.35 (0.31, 0.39)
Heart (% BW)	0.57 (0.55, 0.59)	0.59 (0.56, 0.63)	0.54 (0.49, 0.59)	0.56 (0.52, 0.61)
Hematology				
RBC (cells/μL)	9.71 (9.40, 10.03)	9.81 (9.39, 10.23)	9.94 (9.57, 10.3)	9.91 (9.35, 10.5)
Hgb (g/dL)	14.3 (13.8, 14.8)	14.4 (14.1, 14.8)	14.5 (14.0, 14.9)	14.3 (13.8, 14.9)
Hct (%)	45.9 (44.4, 47.4)	46.4 (45.0, 47.8)	46.7 (45.1, 48.3)	46.6 (44.7, 48.6)
MCV (fL)	47.3 (46.9, 47.6)	47.6 (46.8, 48.3)	47.0 (47.0, 47.0)	47.0 (45.4, 49.6)
MCH (g/dL)	14.7 (14.6, 14.8)	14.7 (14.3, 15.0)	14.6 (14.4, 14.7)	14.5 (14.1, 14.8)
MCHC (g/dL)	31.2 (30.9, 31.4)	31.0 (30.7, 31.4)	31.0 (30.8, 31.2)	30.7 (30.4, 30.9)
Plt (cells/μL)	1162 (994, 1330)	1032 (879, 1185)	1132 (1036, 1228)	1033 (868, 1198)
WBC (cells/μL)	2.73 (2.07, 3.38)	3.10 (2.04, 4.16)	3.05 (2.29, 3.81)	2.95 (2.07, 3.83)

The results are presented as the mean with 95% CI across 14–15 mice for body weight and 7–8 mice for organ weights and hematological parameters, and are considered statistically significant (†) at $P \leq .05$.

* Experimental group description: AIN-93M (+PK), mice were fed a control diet without rice flakes and supplemented with PK; AIN-93M-RICE (+PK), mice were fed a PK-deficient diet with 50% rice flakes supplemented with PK; AIN-93M-RICE (−PK), mice were fed a PK-deficient diet with 50% rice flakes without PK supplementation; AIN-93M-RICE (−/+PK), mice were fed a PK-deficient diet with 50% rice flakes without PK supplementation for the first 5 weeks and with PK supplementation for the last 5 weeks.Abbreviations: BW, body weight; Hct, hematocrit; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; Plt, platelets; RBC, red blood cell; WBC, white blood cell.

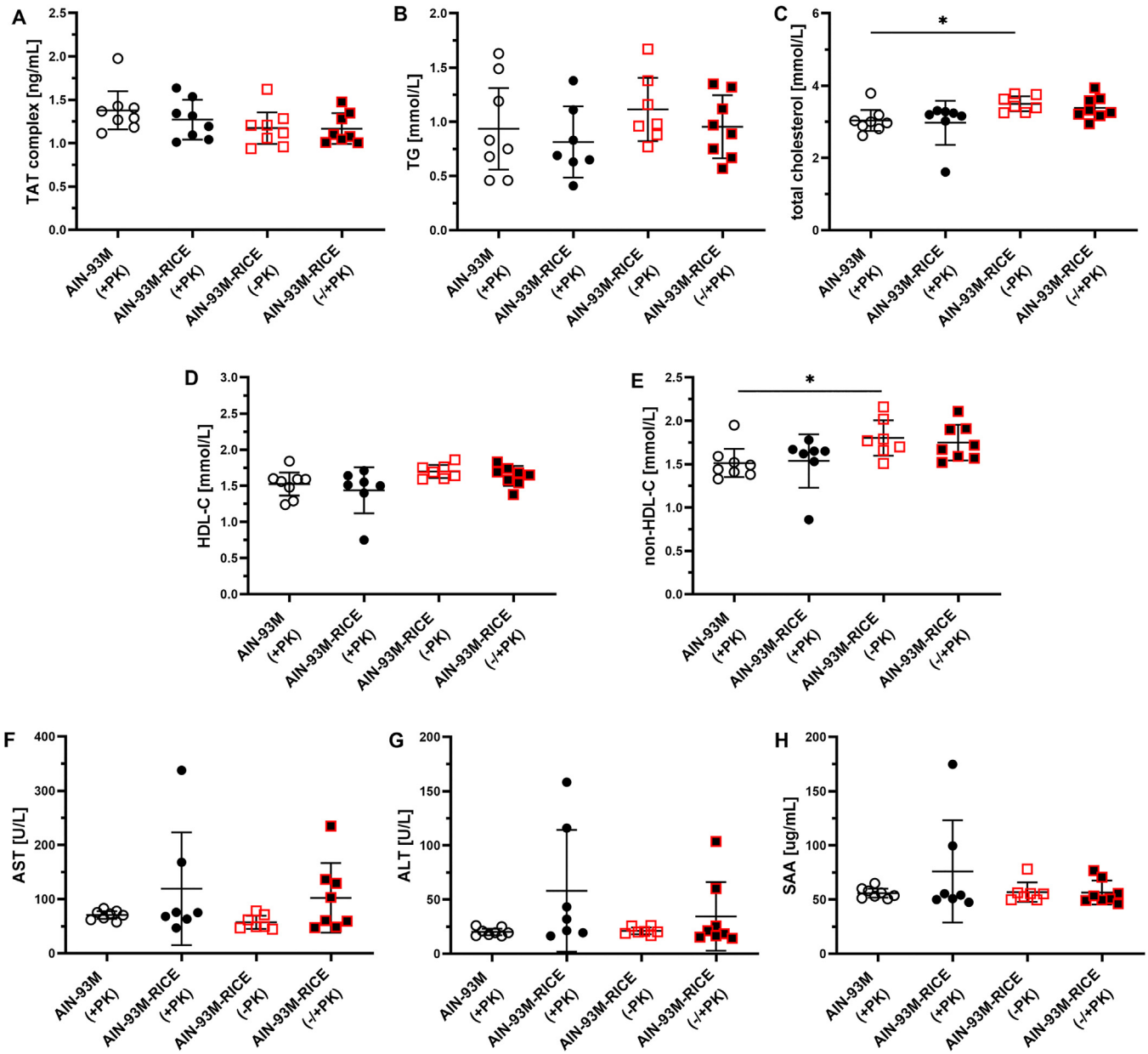

Fig. 2. Lack of effects of dietary phylloquinone deficiency on coagulation and basic biochemical parameters in normolipidemic mice. Coagulation system activity was evaluated based on thrombin-antithrombin (TAT) complex concentrations (A) determined in citrated murine plasma. Lipid profiles (B-E) and the levels of the liver enzymes aspartate aminotransferase (AST; F) and alanine aminotransferase (ALT; G), and serum amyloid-A (SAA; H) were measured in murine plasma collected using K_2EDTA. The results are presented as the mean with 95% CI. Seven to eight mice were examined per experimental group. Key: *, $P \leq .05$; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

with undetectable measured parameter levels or <3 measured values per group were excluded from the statistical analysis.

3. Results

3.1. Effects of dietary phylloquinone deficiency on basic biochemical and hematological parameters

Normolipidemic (C57BL/6JCmd) mice fed a PK-deficient diet for 10 weeks (Table 2) remained in a generally healthy condition without changes in BW (Table 3) or basic hematological parameters (Table 3). Normolipidemic mice also did not show altered thrombin activity, as indicated by the unchanged plasma TAT complex concentration (Fig. 2A). Their plasma levels of TG and HDL-C (Fig. 2B, D) and liver enzymes AST, ALT, and SAA (Fig. 2F-H) were also unaffected. However, PK deficiency increased TC and non-HDL-C levels (AIN-93M (+PK) vs. AIN-93M-RICE (−PK): 3.04 vs. 3.50 mmol/L for TC and 1.51 vs. 1.80 mmol/L for non-HDL-C; Fig. 2C, E).

3.2. Effects of dietary phylloquinone deficiency and its supplementation on endothelial function

Normolipidemic mice fed a PK-deficient diet for 5 weeks showed significant and comparable impairment of endothelium-dependent vasodilation (Fig. 3A-C), in the thoracic aorta, abdominal aorta and the femoral artery as indicated by Ach-induced response in the ThA (7.49% in AIN-93M (+PK) vs. −1.35% in AIN-93M-RICE (−PK)) and in the AbA (6.59% in AIN-93M (+PK) vs. −2.35% in AIN-93M-RICE (−PK)) as well as FMD in the FA (35.10% in AIN-93M (+PK) vs. 18.22% in AIN-93M-RICE (−PK)). The SNP-

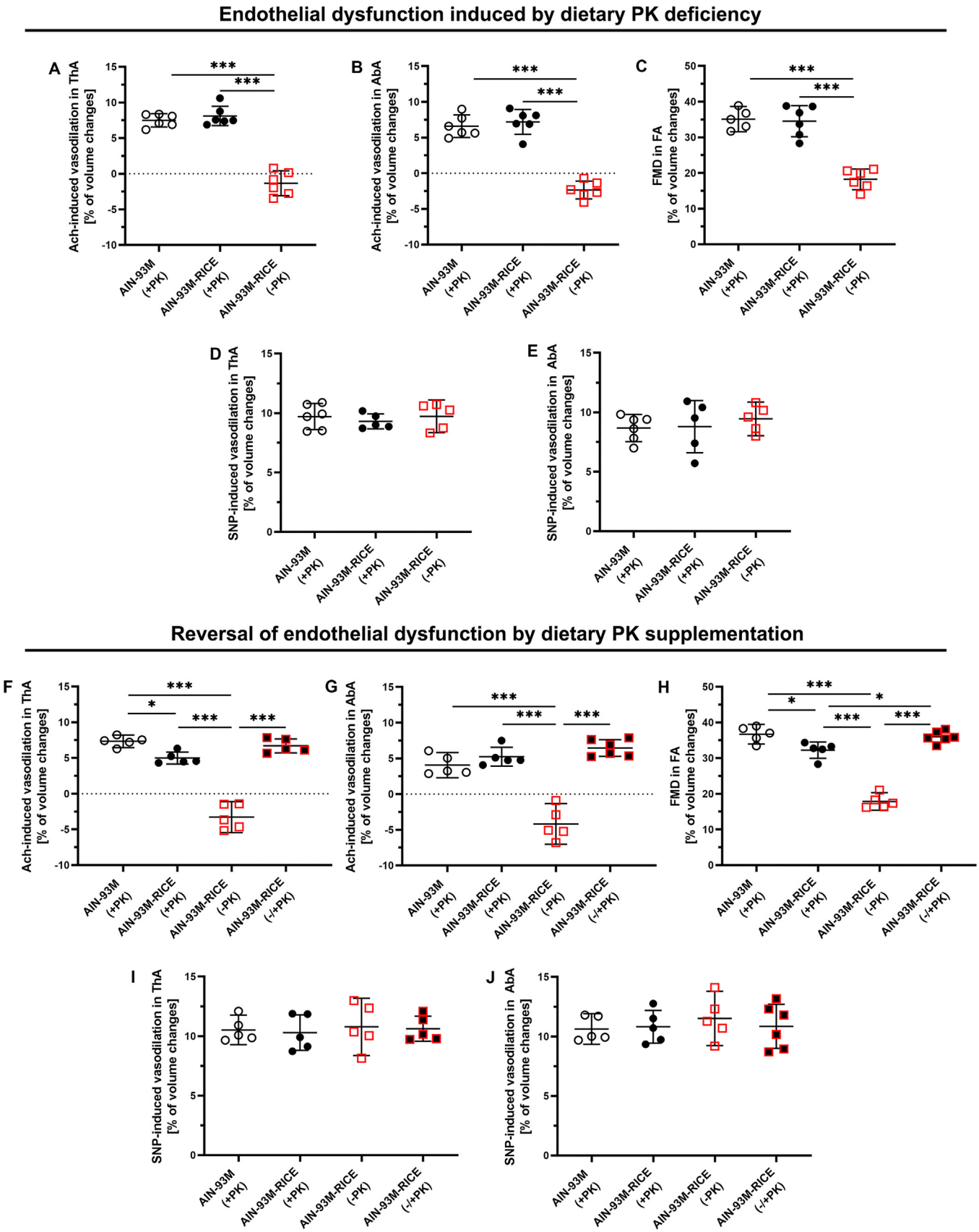

Fig. 3. Detrimental effects of dietary phylloquinone deficiency on endothelial function in normolipidemic mice and its reversal by phylloquinone supplementation. Endothelium-dependent vasodilation was assessed *in vivo* by magnetic resonance imaging (MRI) as the response to acetylcholine (Ach) administration in the thoracic aorta (ThA; A, F) and abdominal aorta (AbA; B, G) and flow-mediated dilatation (FMD) in the femoral artery (FA; C, H). Endothelium-independent vasodilation was evaluated by the response to sodium nitroprusside (SNP) administration in the ThA (D, I) and AbA (E, J). MRIs were performed 5 weeks (A–E) and 10 weeks (F–J) after starting the diet. The results are presented as the mean with 95% CI. Five to six mice were examined per experimental group. Key: *, $P \leq .05$; ***, $P < .001$.

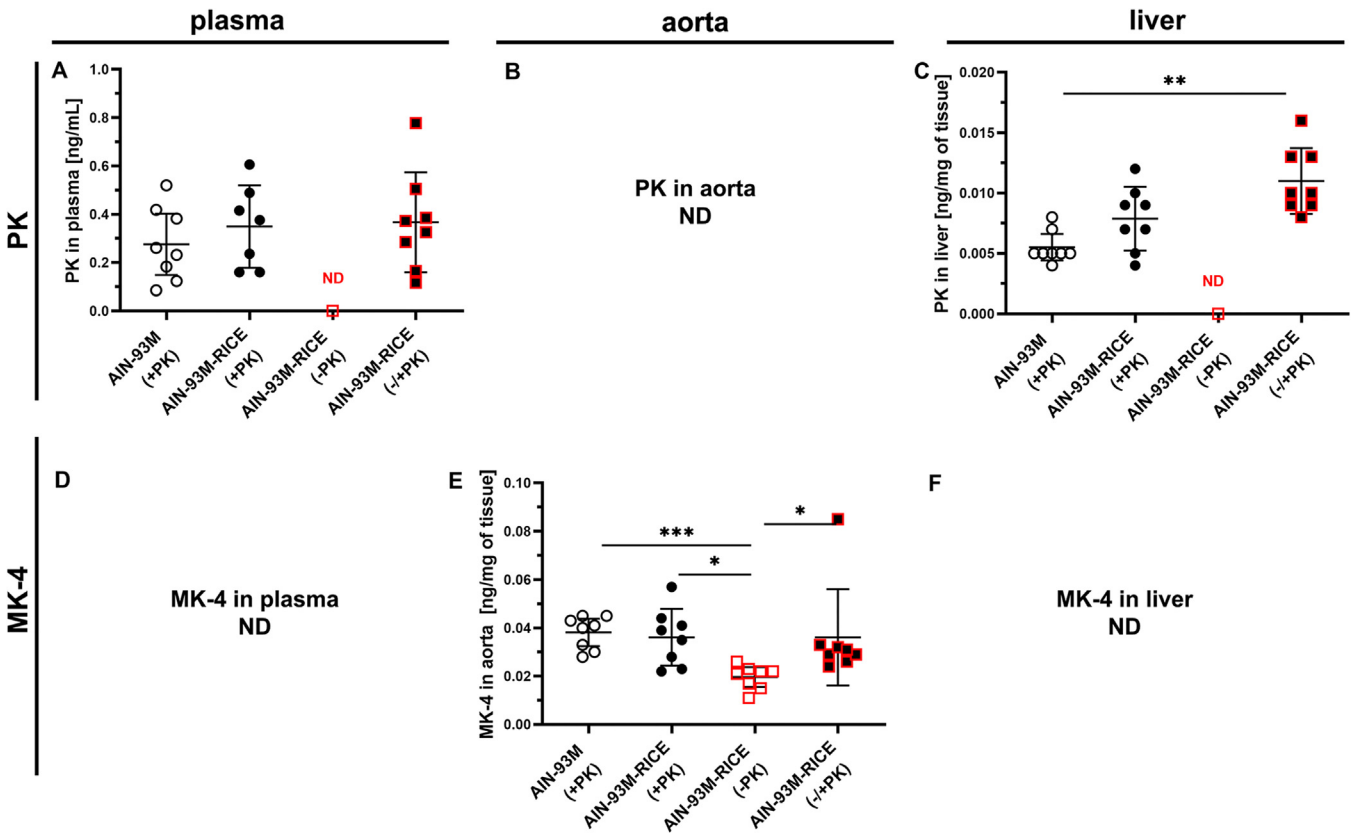

Fig. 4. Effects of dietary phylloquinone deficiency on phylloquinone and menaquinone-4 levels in the plasma, aorta and liver in normolipidemic mice. PK and MK-4 concentrations were determined in murine plasma (A, D), aorta (B, E), and liver (C, F). The results are presented as the mean with 95% CI. Six to eight mice were examined per experimental group. Key: *, $P \leq .05$; **, $P < .01$; ***, $P < .001$; ND, not detectable.

induced endothelium-independent vasodilation in the ThA and AbA was unaffected by dietary PK deficiency (Fig. 3D, E). Subsequently feeding normolipidemic mice fed a PK-deficient diet with a PK-sufficient diet for the 5 weeks completely reversed the endothelial dysfunction induced by dietary PK deficiency (Fig. 3F-H), as indicated by an improved Ach-ThA response (from −3.27% in AIN-93M-RICE (−PK) to 6.69% in AIN-93M-RICE (−/+PK)), Ach-AbA response (from −4.18% in AIN-93M-RICE (−PK) to 6.45% in AIN-93M-RICE (−/+PK)), and FMD-FA response (from 17.86% in AIN-93M-RICE (−PK) to 35.96% in AIN-93M-RICE (−/+PK)). These vasodilatory responses were comparable to those achieved in mice fed the control diet. Mice fed the PK-sufficient AIN-93M-RICE (+PK) diet containing 50% rice flakes had normal endothelial functions in the ThA, AbA, and FA comparable to mice fed the control AIN-93M (+PK) diet without rice flakes but with a similar PK content, further supporting the conclusion that the observed differences in endothelial function were due to PK content not to other factors.

Furthermore, the plasma NO_3^- level was lower in mice with dietary PK deficiency (23.65 μM in AIN-93M (+PK) vs. 10.99 μM in AIN-93M-RICE (−PK)) and reversed by PK supplementation (18.17 μM in AIN-93M-RICE (−/+PK); Supplementary Fig. 1B). While comparing two experimental groups, namely AIN-93M (+PK) vs. AIN-93M-RICE (−PK), aortic NO production assessed *ex vivo* by EPR was significantly reduced in mice with dietary PK deficiency (Supplementary Fig. 1E), and lower NO production was reversed by PK supplementation in AIN-93M-RICE (−/+PK) group (Supplementary Fig. 1D). In contrast, aortic NOS-3 expression assessed by immunohistochemistry was not changed in mice with dietary PK deficiency Supplementary Fig. 1F, G), and p-NOS-3 did not show consistent results (Supplementary Fig. 1H, I). PGI_2 biosynthesis, as indicated by plasma 6-keto-$PGF_{1\alpha}$ levels (Supplementary Fig. 2A), and pro-inflammatory factors measured in aorta, including vWF, ICAM-1, and VCAM-1 were also not significantly altered in either experimental group (Supplementary Fig. 2B-D).

3.3. Effects of dietary phylloquinone deficiency and its supplementation on phylloquinone liver content and intravascular menaquinone-4 biosynthesis

Dietary PK deficiency significantly decreased liver PK levels in normolipidemic mice below the detection limit (<0.17 pg/mg), with a similar effect observed in plasma (<0.05 ng/mL; Fig. 4A, C). In contrast, plasma and liver MK-4 levels were undetectable in all experimental groups (<0.05 ng/mL and <0.17 pg/mg, respectively; Fig. 4D, F). The PK level was undetectable in the aorta (<3.57 pg/mg; Fig. 4B), but the aortic MK-4 level was quantifiable and reduced by dietary PK deficiency (0.038 ng/mg of tissue in AIN-93M (+PK) vs. 0.020 ng/mg of tissue in AIN-93M-RICE (−PK); Fig. 4E). The reduced plasma and liver PK levels and aorta MK-4 levels in mice due to dietary PK deficiency were fully restored after 5 weeks of PK supplementation at ~91.2 μg/kg BW per day (see Section 2.1), reaching 0.37 ng/mL for plasma PK, 0.011 ng/mg of tissue for liver PK, and 0.036 ng/mg of tissue for aortic MK-4 in the AIN-93M-RICE (−/+PK) experimental group (Fig. 4).

Incubation of the aorta with PK increased the intravascular content of PK to detectable levels and increased the content of MK-4, indicating PK uptake by the vascular wall (Fig. 5A) and its intravascular conversion to MK-4 (Fig. 5B). In addition, PK-2,3-epoxide levels were increased and quantifiable in the aorta incubated with PK

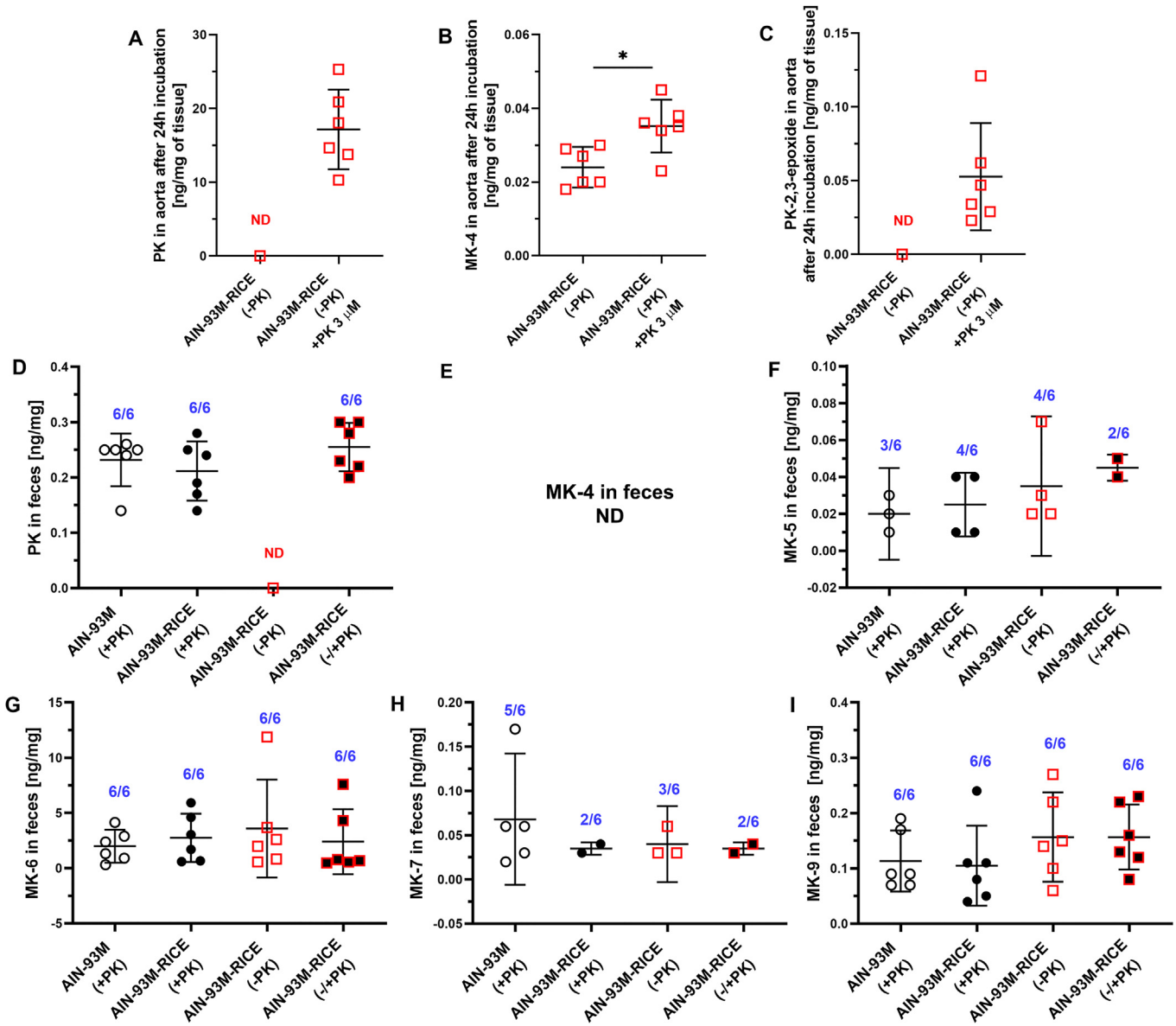

Fig. 5. Conversion of phylloquinone to menaquinone-4 in the vascular wall *ex vivo* and lack of effects of dietary phylloquinone deficiency on menaquinone biosynthesis by gut microbiota in normolipidemic mice. Phylloquinone (PK) uptake (A, C) and conversion to menaquinone-4 (MK-4; B) in the vascular wall were evaluated *ex vivo* after a 24-h incubation of aorta collected from mice fed a PK-deficient diet with 3 μM PK. Vitamin K biosynthesis by gut microbiota was assessed based on PK, MK-4, MK-5, MK-6, MK-7, and MK-9 levels in murine feces (D-I). Since the MK content in feces was not quantifiable in all samples, the number of analyzed samples per group is shown in blue. Six mice were examined per experimental group. The results are presented as the mean with 95% CI. Key: *, $P \leq .05$; ND, not detectable.

(Fig. 5C), while MK-4 2,3-epoxide remained undetectable (<71.43 pg/mg of tissue).

3.4. Effects of dietary phylloquinone deficiency on menaquinones production by gut microbiota

In murine feces, MK-6 and MK-9 had the highest levels, while MK-4, MK-5, and MK-7 were either undetected or were near the detection limit (0.50, 5.00, and 2.50 pg/mg, respectively; Fig. 5E-I). The PK-deficient diet did not significantly alter fecal MK-6, MK-9, MK-5, or MK-7 levels. PK was quantifiable in fecal samples from mice fed a PK-sufficient diet but not detected in fecal samples from mice fed a PK-deficient diet (<0.50 pg/mg; Fig. 5D).

Dietary PK deficiency did not significantly alter the murine gut microbiome composition (Fig. 6 and Supplementary Fig. 3). The culturing method indicated that the most abundant phyla were, in decreasing order, *Firmicutes, Bacteroidota, Proteobacteria*, and *Actinobacteria*. The NGS analysis indicated that the most abundant phyla were *Bacteroidota, Firmicutes, Proteobacteria*, and *Actinobacteria*. The lower abundance of the *Bacteroidota* phylum with the culturing method compared to the NGS method could reflect the difficulties in culturing living *Bacteroidota* bacteria, which did not affect the NGS analysis since it examined all DNA in the sample, including that from nonliving bacteria. The detailed taxonomical classification of the gut microbiome of each experimental group is shown in Supplementary Tables 1 and 2.

3.5. Effects of dietary phylloquinone deficiency on endothelial function in dyslipidaemic mice

The dyslipidaemic E3L.CETP mice with hypercholesterolemia and hypertriglyceridemia (Supplementary Fig. 4C-F) showed pre-

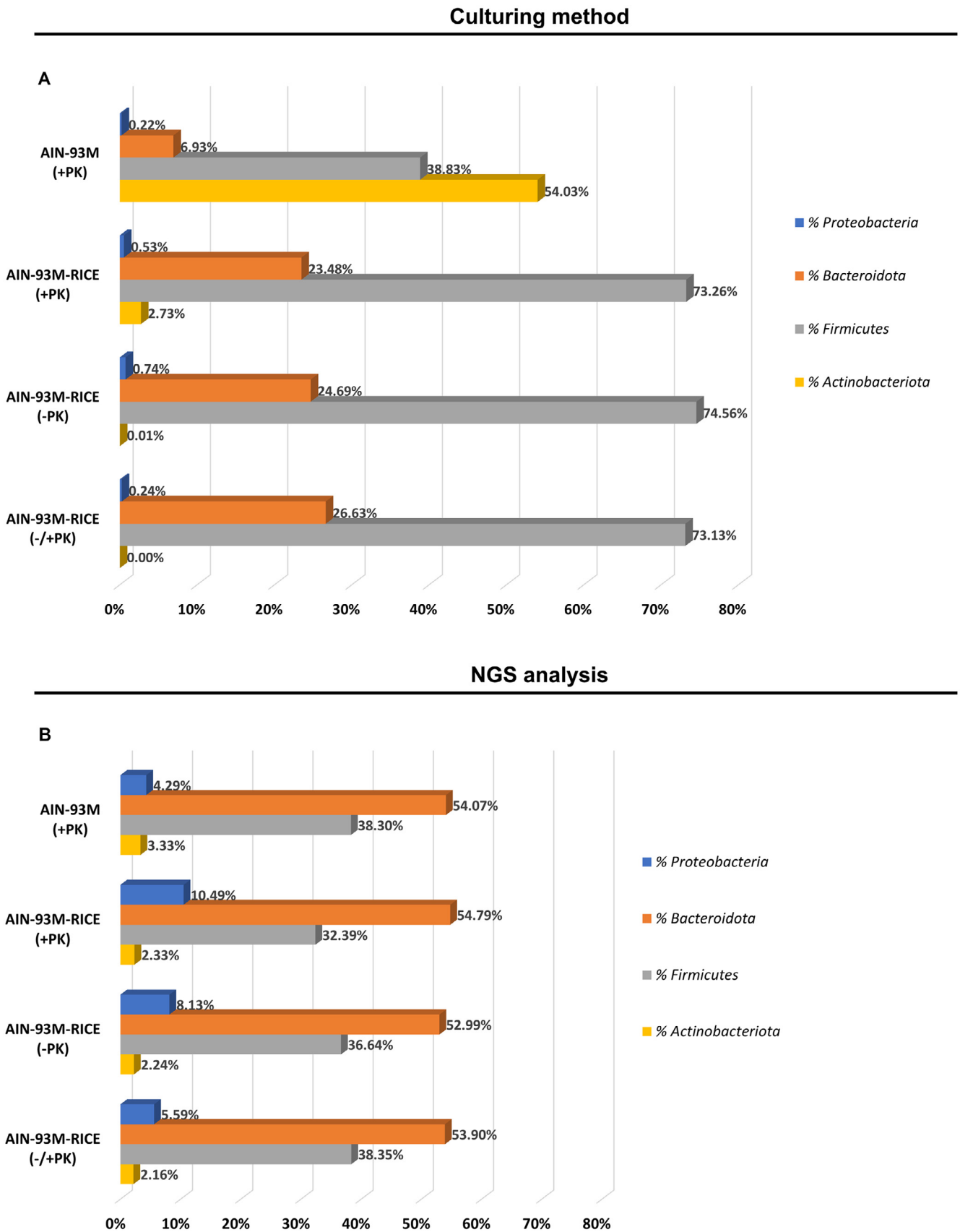

Fig. 6. Lack of effects of dietary phylloquinone deficiency on murine gut microbiota composition at the phylum level in normolipidemic mice. Gut microbiota composition was evaluated using the culturing method as a fraction of that particular phylum in the total number of colonies (A) and by next-generation sequencing (NGS) analysis expressed as a relative abundance percentage (B). Six mice were examined per experimental group.

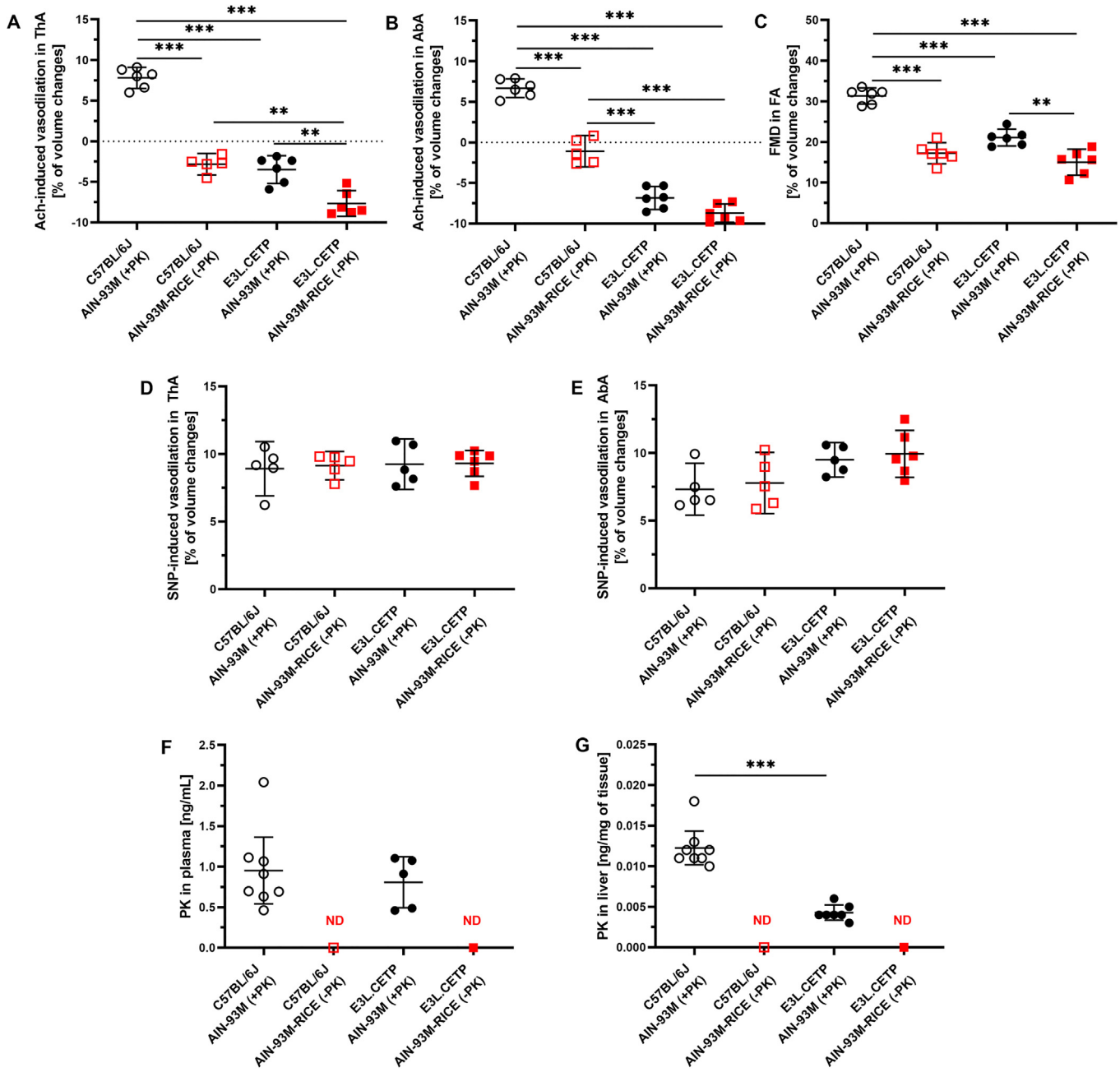

Fig. 7. Detrimental effects of dietary phylloquinone deficiency on endothelial function in dyslipidaemic mice. Endothelium-dependent vasodilation was assessed *in vivo* by magnetic resonance imaging (MRI) as the response to acetylcholine (Ach) administration in the thoracic aorta (ThA; A) and abdominal aorta (AbA; B) and flow-mediated dilataion (FMD) in the femoral artery (FA; C). Endothelium-independent vasodilation was evaluated as the response to sodium nitroprusside (SNP) administration in the ThA (D) and AbA (E). MRIs were performed 10 weeks after starting the diet. The PK concentration was determined in murine plasma (F) and liver (G). The results are presented as the mean with 95% CI. Five to eight mice were examined per experimental group. Key: **, $P<.01$; ***, $P<.001$; ND, not detectable.

existing endothelial dysfunction (Fig. 7), as indicated by impaired endothelium-dependent vasodilation in the thoracic aorta (Fig. 7A) and abdominal aorta (Fig. 7B) and the femoral artery (Fig. 7C), while endothelium-independent vasodilation in the thoracic and abdominal aorta in response to SNP was fully preserved (Fig. 7D, E). Feeding the dyslipidaemic mice a PK-deficient diet for 10 weeks further impaired their endothelial function (Fig. 7A-C), as indicated by further deterioration of the Ach-ThA response (from −3.49% in E3L.CETP AIN-93M (+PK) to −7.66% in E3L.CETP AIN-93M (−PK) and FMD-FA response (from 21.09% in E3L.CETP AIN-93M (+PK) to 15.02% in E3L.CETP AIN-93M (−PK)).

Similarly to normolipidemic mice, dietary PK deficiency did not affect coagulation processes in dyslipidaemic mice, as indicated by unchanged plasma TAT complex concentrations (Supplementary Fig. 4A) and thrombin production assessed by CAT with the lag-time parameter (Supplementary Fig. 4B).

Notably, liver PK basal levels were significantly lower in dyslipidaemic mice (0.004 ng/mg of tissue in E3L.CETP AIN-93M (+PK)) than in normolipidemic mice (0.012 ng/mg of tissue in C57BL/6J AIN-93M (+PK); Fig. 7G), while their plasma PK levels were comparable (Fig. 7F). When dyslipidaemic mice were fed the PK-deficient diet they displayed significantly lower and undetectable

liver and plasma PK levels (Fig. 7F, G), similar to normolipidemic mice fed the PK-deficient diet.

3.6. Effects of dietary phylloquinone deficiency on aortic gamma-glutamyl carboxylase expression in normolipidemic and dyslipidaemic mice

Dietary PK deficiency did not significantly alter aortic GGCX protein expression in normolipidemic mice (Supplementary Fig. 5A). Aortic GGCX protein expression was lower in dyslipidaemic mice than in normolipidemic mice but was unaffected by dietary PK deficiency itself (Supplementary Fig. 5B). The aortic expression of UBIAD1 was also not altered by PK deficiency (Supplementary Fig. 5C, D).

4. Discussion

In this study, we experimentally demonstrated that dietary PK maintains endothelial function. To our knowledge, we reported here for the first time that dietary PK deficiency in normolipidemic mice impaired endothelium-dependent vasodilation and exacerbated preexisting endothelial dysfunction in dyslipidaemic E3L.CETP mice. Importantly, dietary PK deficiency-induced endothelial dysfunction in normolipidemic mice was completely restored by PK supplementation. Since the primary source of PK in humans is diet, and in the general population a low plasma PK level might be a frequent phenomenon [17,44] correlating with cardiovascular risks in some patient groups [19,20], we conclude that adequate dietary PK intake is essential for maintaining healthy endothelial function under normolipidemic and dyslipidaemic conditions. Therefore, ensuring adequate dietary PK intake or considering therapeutic supplementation with exogenous PK might represent an effective approach to support endothelial function in humans.

In our study, a controlled state of dietary PK deficiency in mice was induced by a specially designed PK-deficient diet based on previous studies [33,34], that resulted in endothelial dysfunction. Interestingly, dietary PK deficiency impaired vasodilation in response to Ach in the ThA and AbA and the FMD response in the FA to similar degrees, unlike other vascular pathologies such as those induced by a high-fat diet [25] and chronic heart failure [40], showing a heterogeneity in vascular functional responses. Here, dietary PK deficiency induced endothelial dysfunction equally in the ThA, AbA, and FA with a preserved vascular SNP response, that indicates the importance of PK specifically in maintaining endothelium-dependent vasodilation in distinct parts of the vascular tree. The adverse effect of dietary PK deficiency on endothelial function in mice may be attributed directly to insufficient PK content in the diet since PK supplementation fully restored endothelial function to physiological baseline levels. To extend our finding on the effects of dietary PK deficiency on endothelial function found in normolipidemic mice to dyslipidaemic condition, we used humanized murine model of dyslipidemia represented by E3L.CETP mice [27–30]. Our study shows that dietary PK deficiency exacerbated pre-existing endothelial dysfunction in E3L.CETP mice, indicating the importance of PK in maintaining endothelial function not only in normolipidemic but also in dyslipidaemic conditions. Our results are further supported by a recent report indicating that PK supplementation in ApoE/LDLR$^{-/-}$ mice, another model of dyslipidemia, improved endothelial function [45] reinforcing the notion that PK maintains endothelial function also in dyslipidaemic conditions.

Impaired endothelium-dependent vasodilation *in vivo* induced by dietary PK deficiency was associated with reduced plasma NO_3^- levels, a phenomenon that was completely restored by PK supplementation suggesting that impaired NO-dependent function by dietary PK deficiency was compensated by activation of the NO_3^- reductive pathway to increase systemic NO bioavailability [40]. NO production in aorta assessed *ex vivo* by EPR was also reduced by dietary PK deficiency, but this difference was observed only when two experimental groups were compared (Supplementary Fig. 1E). Aortic expression of NOS-3 remained unchanged, and phosphorylated NOS-3 did not show consistent changes in dietary PK deficiency group. The results showing clear-cut changes in functional NO-dependent response *in vivo*, that were not associated with significant changes in plasma nitrite levels, aortic NO production, or NOS-3 expression seem to be compatible with our previous studies. Indeed, in other murine models of endothelial dysfunction, MRI-based functional assays for testing NO-dependent vasodilation *in vivo* was more sensitive in detecting endothelial dysfunction than plasma NO metabolites measurements, *ex vivo* production of NO in aorta or vascular NOS-3 expression [25,40].

Our results showing PK-mediated improvement in NO-dependent endothelial function stay in contrast with some previous reports indicating that PK impaired NO-dependent vasodilation *ex vivo* in rat carotid arteries [46,47], and impaired NO bioavailability [46]. In addition, Nolan *et al.* showed that PK inhibited vasoactive PGI_2 and prostaglandin E_2 biosynthesis *in vitro* in bovine aortic endothelial cells [48], while in our study PGI_2 biosynthesis was not affected by dietary PK deficiency (Supplementary Fig. 2A). However, our study is compatible with a previous report showing that high dose of PK activated NOS pathway resulting in hypotension in rats *in vivo* [49], and improved NO-dependent function in ApoE/LDLR$^{-/-}$ mice *in vivo* [45]. Altogether, it seems that the *in vivo* vascular effects of PK on NO-dependent function cannot be fully recapitulated *in vitro*, in cultured endothelial cells, for example due to the detrimental pro-oxidative effects of menadione present in micromolar concentrations under *in vitro* experimental conditions [3,46].

Although, we clearly demonstrated the role of PK in maintaining endothelial function, we did not determined mechanisms involved, which can be mediated either by vitamin K-dependent proteins (VKDPs) or noncanonical pathways. Mechanisms of extrahepatic vitamin K activity were previously associated with vitamin K-dependent carboxylation of matrix Gla protein (MGP), osteocalcin (OS), growth arrest-specific 6 (GAS6), periostin (POSTN), and others [12,13,50]. Indeed, there is evidence that VKDPs regulated by MKs [51–54] could also be targeted by PK [51,52,54,55]. In our study, vascular GCCX protein expression was not consistently changed by a PK-deficient diet (Supplementary Fig. 5A, B), that partially could exclude the involvement of vascular VKDPs in endothelial function regulation by PK. Vitamin K can also act in extrahepatic tissues via noncanonical mechanisms independent of VKDPs, such as regulating transcription factors [steroid and xenobiotic receptor (SXR) and nuclear factor kappa B (NF-κB)] [36,53], regulating electron carriers in mitochondria [11,56], or suppressing ferroptosis [57] via the ferroptosis suppressor protein 1 (FSP1)-dependent noncanonical vitamin K cycle [58] with the involvement of VKORC1L1 [59]. Furthermore, vitamin K displayed protective effects in endothelium via antisenescence effects attributed to decreasing DNA damage and the modulation of NF-κB activation [45]. Interestingly, in our work, the aortic CoQ10 level was decreased in mice fed a PK-deficient diet (Supplementary Fig. 6B), potentially suggesting oxidative stress [60], NOS-3 uncoupling [61], or the compensatory utilization of CoQ10 to replace PK in noncanonical mechanisms [56,58]. On the other hand, we observed neither activation of vascular oxidative stress measured as aortic production of H_2O_2 (Supplementary Fig. 7), nor endothelial inflammation assessed by the expression of ICAM-1 and VCAM-1 in aorta (Supplementary Fig. 2C, D). Accordingly, further studies are needed

to identify the biochemical pathway responsible for the effect of PK on NO-dependent function demonstrated here. However, short-term dietary PK deficiency inducing endothelial dysfunction was not associated with coagulation disorders, unlike in some previous studies with dietary PK deficiency models [34] or pharmacologically induced vitamin K deficiency by vitamin K antagonists [62], also in germ-free animals [63,64] and animals kept in metabolic cages with limited coprophagy [65]. It is highly probable, that in our model of PK deficiency, as we did not limit coprophagy, coagulation was maintained by gut microbiota-derived MKs, which, like PK, can post-translationally regulate coagulation factor synthesis in the liver [66]. Therefore, using this experimental approach, we demonstrated the specific endothelial effects of short-term dietary PK deficiency that were unrelated to the overt functional deficiency of coagulation factor synthesis in the liver supported by vitamin K, either by PK or by MKs.

In the present work, we were not able to finally determine, whether the regulation of NO-dependent endothelial function was achieved by direct PK action or indirect action of PK via intravascular MK-4 synthesis. PK deficiency could possibly limit vascular capacity to produce MK-4, that similarly to MK-7, improves NO-dependent function [35,45]. While it was previously reported that dietary PK could be converted to MK-4 in the intestine [67], brain [68], kidney [69], macrophages [36], or aorta [70] our findings extend this knowledge by showing that the aortic MK-4 level was decreased in mice fed PK-deficient diet *in vivo*, and this fall was fully reversed by PK supplementation. Interestingly, UBIAD1 expression in aorta remained unchanged in response to dietary PK deficiency (Supplementary Fig. 5C, D). However, we directly showed that exogenous PK was converted into endogenous MK-4 in aorta *ex vivo*, indicating that endogenous MK-4 can be generated from exogenous PK in the aorta. Accordingly, endogenous MK-4 formed after intravascular conversion of PK could be responsible for the beneficial effect of exogenous PK supplementation, but the direct vascular PK activity cannot be excluded. Altogether, we are tempted to speculate that both PK and MKs may support vascular homeostasis, and their relative roles might be related to their availability in endothelium and vascular wall. Of note, MK-4 biosynthesis from PK-derived menadione generated in the intestine or vasculature should be also considered [71,72].

Yet, we excluded the role of endogenous microbiota-derived MKs in the vascular effects of exogenous PK since the composition of gut microbiota and gut microbiota-derived MKs levels measured in feces were unchanged by a PK-deficient diet, and importantly did not contain significant MK-4 levels. The most abundant MKs in feces was MK-6, and the sum of microbiota-derived MKs in feces was much above the level of PK in mice fed PK-sufficient diet. Despite this, MK-6 and other long-chain MKs derived from microbiota could not replace PK action to maintain endothelial function.

In our study, when E3L.CETP mice were fed a PK-sufficient diet, plasma PK levels were preserved but in their liver PK levels were reduced. These findings most likely reflected a delayed ApoE- and LDLR-mediated clearance pathway for non-HDL-C lipoproteins in this model [73], a major pathway delivering PK to the liver. Given the delayed ApoE- and LDLR-mediated clearance observed in patients with familial dysbetalipoproteinemia carrying the APOE*3-Leiden variant of the APOE gene [29,30,74,75], which may also occur in any form of aberrant ApoE-mediated liver lipoprotein uptake, it might well be that humans with these conditions have deficient liver PK content but normal plasma PK content. If so, it is tempting to speculate that prolonged low dietary PK intake may impair the liver's buffering capacity to maintain vascular PK delivery.

Importantly, our results suggesting that dietary PK maintains endothelial function are consistent with clinical studies reporting that a low level of circulating PK was associated with vascular pathophysiological states, including increased CVD risk and coronary artery calcification progression in patients treated for hypertension [19,20], and a higher rate of all-cause mortality [76]. Furthermore, our results suggest that improved endothelial function due to PK intake could have contributed to the overall improvement in vascular health previously described in various clinical trials with PK intake [16,21,51,77].

Obviously, clinical studies in humans are needed to provide unequivocal evidence that dietary PK maintains endothelial function in normolipidemic and dyslipidaemic humans, and to find out the optimal dose of PK to maintain endothelial function in patients. PK nutritional requirement set to 1 μg/kg BW by European Food Safety Authority (EFSA) [2] was established based on its role in coagulation processes and may be too low for optimal PK effects on vascular health [52,78,79] as also suggested recently by Schultz *et al.* [18]. Given that low plasma PK concentrations in humans were associated with higher cardiovascular risk [19,20], preserving endothelial and vascular health might indeed require higher PK doses than the established human PK nutritional requirement that is most likely met in the general population and should be reconsidered [2].

Of note, in our work, basal plasma concentration of PK in mice fed PK-sufficient diet was in the range 0.28 ng/mL to 0.35 ng/mL, and after reversal of PK deficiency was equal to 0.37 ng/mL; the endothelial dysfunction was present when PK plasma level was very low but preserved when PK levels were ~0.3 ng/mL. In humans, a wide variation in plasma PK concentrations, was reported ranging from 0.22 to 3.95 nmol/L (equal to 0.10–1.78 ng/mL) [44], suggesting that lower plasma PK levels may be quite common [44]. Importantly, Wei *et al.* demonstrated that low plasma PK (<0.26 ng/mL; quartile 1) was associated with a significantly higher risk of ischemic stroke as compared with patients with higher plasma PK (≥1.08; quartile 4) [80]. The most striking example of deficient vitamin K intake could be related to hemodialysis situation associated with low PK level [81], that could contribute to endothelial dysfunction and cardiovascular pathology, consistent with the beneficial cardiovascular effects of PK supplementation in patients with chronic kidney disease [82,83]

Taken together, our study expanded the well-known list of dietary PK deficiency consequences [3] with a new element, namely impaired NO-dependent endothelial function. While endothelial function is not measured routinely in patients, there is overwhelming evidence that impaired endothelial-dependent vasodilation is the early hallmark of CVDs [22], and improved endothelial function reduces cardiovascular morbidity and mortality [84]. Therefore, returning to the seminal concept of Casimir Funk, elaborated nearly 100 years ago, identifying dietetic factors whose deficiency causes 'deficiency disorders', such as beriberi, scurvy, rickets, and pellagra [85,86], we suggest that inadequate dietary intake of PK, the primary source of vitamin K in humans, may lead to endothelial dysfunction. Therefore, PK has joined the group of vitamins, their metabolites, and nutraceuticals preserving endothelial function [87–90].

In conclusion, this study showed that PK plays a vital role in maintaining NO-dependent endothelial function in normolipidemic and dyslipidaemic mice either via the direct action of exogenous PK-mediated activity or intravascularly biosynthesized MK-4. However, MKs synthesized by gut microbiota were not involved. Further studies are needed to determine the specific mechanisms involved in PK-associated endothelial protection and whether the PK-induced improvement in NO-dependent endothelial function shares the same mechanism as MKs [35]. Whatever the mechanistic conclusion is, the improvement in NO-dependent endothelial function associated with PK intake clearly emphasizes the role of

dietary PK in maintaining vascular health. Accordingly, PK given as a nutritional or therapeutic regimen may improve NO-dependent endothelial function in the general population and patients with CVD or other conditions with low plasma PK levels and impaired endothelial function.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2025.109867.

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