Regular Article

Atheroprotective effect of dietary walnut intake in ApoE-deficient mice: Involvement of lipids and coagulation factors

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ABSTRACT

Introduction: Consumption of n-3 polyunsaturated fatty acids (PUFA) and antioxidant polyphenols is considered to decline the risk of cardiovascular disease.
Materials and Methods: To provide an explanation for this cardioprotective effect, we performed an intervention study with proatherogenic $Apoe^{-/-}$ mice which were fed during eight weeks with a high fat diet supplemented with either walnuts (rich in n-3 PUFA and antioxidant compounds), walnut oil (with n-3 PUFA only) or sunflower oil as a control (12 mice per group).
Results: Feeding walnuts, but not walnut oil, caused a 55% reduction in atherosclerotic plaque development in the aortic arch in comparison to the control diet. This was associated with reduced staining of plaques for CD36, a scavenger receptor expressed by macrophages. Feeding mice with walnuts also lowered plasma levels of triglycerides, cholesterol and prothrombin with 36%, 23% and 21 %, respectively, compared to control diet. In addition, accumulation of lipids in the liver was decreased, while plasma antioxidant capacity was increased. On the other hand, feeding mice with walnut oil did not provoke significant changes in these parameters in comparison to the control diet. Platelet activation and thrombus formation under flow remained unchanged with either diet.
Conclusions: In $Apoe^{-/-}$ mice on high fat diet, intake of dietary walnut (but not walnut oil) beneficially influences lipid metabolism and atherosclerotic plaque development, with no more than limited effects on platelet and coagulation function.

Introduction

In today's world, cardiovascular disease, in particular atherothrombosis is a major cause of morbidity and death [1]. Both atherosclerosis and atherothrombosis are multifactorial disorders, in which an altered lipid metabolism, activation of platelets and the coagulation system, and inflammatory processes play predominant roles. The development of atherosclerotic lesions is markedly promoted by interactions of plasma lipids, platelets and coagulation factors with the affected arterial vessel wall [2–4]. In addition to lipid-lowering and antithrombotic medication, there is recurrent interest in the finding of nutritional components that can reduce atherosclerotic plaque development and suppress eventual thrombotic complications.

Epidemiologic studies have shown significant associations between the intake of n-3 polyunsaturated fatty acids (PUFA) or antioxidants, as present in fish oil, and diminished risk of cardiovascular disease [5–8]. Mice may provide a suitable animal model to study the dietary effects of fish oil, as it was found that fish oil feeding lowers the plasma levels of lipids and coagulation factors in hyperlipidemic mice, such in agreement with an antithrombotic effect of n-3 PUFA [9]. Another rich source of both n-3 PUFA and antioxidant polyphenols are walnuts (*Juglans regia* L.), which contain the highest content of α-linolenic acid among all edible plants [10–14]. In a limited number of human studies, health-promoting effects of walnut consumption have been reported [15,16]. Individual papers yet disagree whether [17] or not [18] walnut intake affects plasma lipid levels and platelet activation parameters.

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In this paper, we hypothesized that walnut intake, due to the combined presence of n-3 PUFA and antioxidants, has a cardioprotective effect by interfering with the atherosclerotic process and hemostatic reactions. To investigate this, we performed an experimental intervention study with proatherogenic $Apoe^{-/-}$ mice, which were fed Western-type, high fat and cholesterol diets containing whole walnuts or purified walnut oil (rich in n-3 PUFA), such in comparison to a high fat control diet containing purified sunflower oil (rich in n-6 PUFA). We determined the dietary effect on atherosclerotic plaque development as primary endpoint, and compared this with the effects on lipid metabolism and on platelet and coagulant functions.

Materials and Methods

Materials

Ketamine and xylazine were obtained from Eurovet (Bladel, The Netherlands). H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla CA, USA). Murine PAR4 peptide (GYPGKF) was a gift from Dr. S. Kunapuli, Temple University (Philadelphia PA, USA). Low molecular weight heparin (fragmin) was from Pfizer (Capelle a/d IJssel, The Netherlands). Convulxin was purified to homogeneity from the crude venom of *Crotalus durissus terrificus* (Latoxan, Valence, France) [19]. Recombinant human tissue factor was from Dade Behring (Marburg, Germany); Horm type I collagen from Nycomed Pharma (Munich, Germany); fluorescein isothiocyanate (FITC) labeled annexin A5 from PharmaTarget (Maastricht, The Netherlands). FITC-labeled anti-CD62 mAb was from Immunotech (Marseille, France); phycoerythrin (PE)-labeled anti-mouse JON/A mAb from Emfret Analytics (Würzburg, Germany); and anti-mouse CD36 mAb from Chemicon (Billerica MA, USA). Other materials were from sources as described before [9,20]. All other chemicals came from Sigma (St. Louis MO, USA).

Animals and Diets

Power calculation was performed for calculating the required number of mice/group to determine dietary effects on plaque size. For calculating group size (N), the equation of L. Sachs was used, evaluating the difference between two averages. $N = 2 (z_{\alpha/2} - z_{\pi})^2 \times (\sigma/\delta)^2$, wherein is $2(z_{\alpha/2} - z_{\pi})^2 = F$. For $\alpha = 0.05$ and $\pi = 80\%$, $F_{0.80}$ is tabeled as 15.7. As a consequence, $N = 15.7 \times (\sigma/\delta)^2$, in which the standard deviation $\sigma = 0.047$, and the relevant measured difference in plaque size $\delta = 0.060$ mm^2. This gave a group size N = 9.63 (round up 10), which was corrected for an expected loss of 20%, using the equation N = (a - 0.2a) N, resulting in a required number per group of 12.

Experiments were approved by the local Animal Experimental Committee. Pathogen free $Apoe^{-/-}$ male mice on a C57Bl/6 background were obtained from Charles River (Maastricht, The Netherlands). During the study, animals were kept individually in transparent macrolon cages at 20-22 °C (12 h light/dark cycle; 60% relative humidity). The mice were allowed to drink water *ad libitum* and were provided with 5 g of indicated diets daily. Intake of food was estimated by daily back weighing of uneaten food. Body weight was measured weekly.

During two weeks, 36 mice (aged 8 weeks) were fed a standard purified diet containing 10 energy% (4 weight%) fat from TestDiet (AIN-93 M, Richmond IN, USA). $Apoe^{-/-}$ mice on a low-fat diet do not contain atherosclerotic plaques in the aortic arch at an age of 10 weeks [21]. The animals were then randomly divided into three groups, and fed during eight weeks a high fat diet (5 g food/day), containing 0.2 weight% cholesterol and 34 energy% (20 weight%) fat (Supplementary Table 1). Herein, the standard diet was supplemented with: (i) sunflower oil rich in n-6 PUFA (high fat control (HFC) group); (ii) walnut oil rich in n-3 PUFA (WO group); (iii) whole walnuts rich in n-3 PUFA and other components such as antioxidant polyphenols (WW group). Thus, animals from the WW group received daily 1.2 g homogenized walnuts (*Juglans regia* L.) per 5 g diet, corresponding to an intake of 24 energy%

walnut fat. Other macronutrients, i.e. protein, carbohydrate, and fiber were kept equal in all groups on a weight% base (Supplementary Table 1). Whole walnuts and walnut oil, both from the same batch of nuts, were obtained from Zade Edible Oil Company (Konya, Turkey), as was the sunflower oil. Diets were stored in aliquots at −80 °C. Portions were daily thawed before administration.

At the end of the dietary period, mice were deprived from food for 5 h to obtain sober profiles of blood lipids. Two mice of the WO group died preliminary for unknown reasons. All other 34 mice were used for blood collection and plaque analysis.

Collection of Blood and Tissues

Mice were anesthetized by subcutaneous injection of 0.1 mg/g body weight ketamine and 0.02 mg/g body weight xylazine, and blood was drawn from the retro-orbital plexus. From half of the mice ($n = 5$-6 per group), 0.5 ml blood was collected into PPACK (40 μM, final concentration) plus heparin (5 U/ml) to measure thrombus formation. Remaining blood and blood from the other mice was drawn into trisodium citrate (12.9 mM) to prepare platelets and platelet-free plasma [9,20]. The plasma samples were snap-frozen in liquid nitrogen and stored at −80 °C for later determination of coagulation factors, lipids and thrombin generation. Sufficient plasma for these measurements was obtained from 9 mice in each group. All data obtained are presented in the paper, and no outlier data were removed.

Immediately after bleeding, the animals were sacrificed by diaphragm puncture, after which aortic arches and livers were dissected according to standard protocols [9,22]. The organs were isolated from adherent tissue, and rinsed with ice-cold saline to remove remaining blood. Aortic arches including the main branch points (brachiocephalic trunk, left common carotid artery and left subclavian artery), were carefully removed from surrounding tissue, and fixed in 1% formaldehyde. Main parts of the livers were snap-frozen in liquid nitrogen. Two liver lobes were fixed in 4% formaldehyde. Fixed tissues were embedded into paraffin and sectioned with a rotary microtome and processed, as described elsewhere [23].

Measurement of Lipids and Cholesterol

Lipids in diets were extracted with chloroform/methanol and trans-methylated, after which fatty acid profiles were analyzed by gas chromatography [24]. Plasma levels of total cholesterol (Cholesterol RTU, Biomerieux, France) and triglycerides (Triglyceride/GB GPO-PAP, Roche Diagnostics, Switzerland) were measured with enzymatic colorimetric commercial tests [9]. To measure the lipid content in livers, frozen liver lobes were homogenized in 250 mM sucrose, 2 mM EDTA and 10 mM Tris (pH 7.45) by shaking for 30 s at 5000 rpm in a closed tube [25]. Triglycerides in livers were measured, as described above, and normalized for protein content, as determined with the BCA method (Pierce, Rockford IL, USA).

Lipoprotein cholesterol distribution in plasma was assessed by fast protein liquid chromatography [26,27]. Equal volumes of plasma samples from 10 animals per group were pooled. The lipoproteins were size-fractionated with a Superose 6PC 3.2/30 column (Amersham Biosciences, Roosendaal, The Netherlands).

Plasma levels of triglycerides and cholesterol at baseline, in mice receiving normal chow diet (4 energy% fat, no cholesterol) were determined at 0.64 ± 0.24 mM and 19.90 ± 3.7 mM, respectively (mean $\pm$ sd, $n = 10$).

Coagulation Parameters

Tissue factor-induced thrombin generation was measured in citrate-anticoagulated platelet-free plasma triggered with tissue factor (6 pM) and phospholipids (10 μM), according to the automated thrombogram method adapted for mouse plasma [9,28]. Plasma

levels of prothrombin and factor V were measured with factor-deficient plasmas, as described [9]. Fibrinogen was determined according to the Claus method [29].

Histology and Immunohistochemistry

Paraffin-embedded livers and aortic arches with main branch points were cut into approximately 40 sections, each 4 μm thick, following routine procedures [22]. Series of 20 sections, representing the central area with intact morphology of the complete arch, and serial sections of liver were used for histological analysis. Four representative sections (16 μm apart) of each aorta and of the liver were stained with hematoxylin and eosin. Total plaque area in the aortic sections (2×4 mm) was determined by averaging identified atherosclerotic areas in each of the four sections. CD36 expression in aortic sections was detected by staining with anti-mouse CD36 mAb (1:100).

Flow Cytometry

Whole blood samples were diluted in Tyrode's Hepes buffer (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA, pH 7.45), supplemented with 2 mM $CaCl_2$ and 20 U/ml fragmin. Aliquots were activated for 10 min with ADP (40 μM), convulxin (100 ng/ml) or PAR4 peptide GYPGKF (0.5 mM) without stirring. Activation of platelet integrin $\alpha_{IIb}\beta_3$ and secretion of α-granules was detected with PE-labeled JON/A mAb (1:20) and FITC-labeled mAb against CD62P (1:40), respectively [20]. Fluorescence was measured with a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were analyzed by WinMDI 2.9 software [30].

Thrombus Formation Under Flow

Glass coverslips, coated with fibrillar type I collagen, were blocked with Tyrode's Hepes buffer containing 1% BSA, and mounted into a transparent parallel-plate flow chamber, as described [31,32]. PPACK/heparin-anticoagulated blood was perfused through the chamber at a shear rate of 1000 s⁻¹. During perfusion, high-resolution microscopic images were recorded in real time with a Visitech imaging system (Sunderland, United Kingdom) equipped with two parallel-placed intensified, charge-coupled device cameras for recording brightfield light and epifluorescence. After 4 min, the flow chamber was perfused with Tyrode's Hepes buffer pH 7.45 supplemented with 1 U/ml heparin at the same shear rate for 2 min. Exposure of phosphatidylserine was detected with FITC-labeled annexin A5 (1 μg/ml). Phase-contrast and fluorescent images were taken from at least 10 microscopic fields, which were randomly chosen. Images were analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

Antioxidant Capacity

Diet samples were lyophilized, and extracted with sodium phosphate buffer (aqueous/water soluble antioxidants) or ethanol (organic/ethanol soluble antioxidants). Extracted diet samples and plasmas were deproteinized with trichloro acetic acid, and their Trolox equivalent antioxidant capacity (TEAC) was determined using an ABTS [2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid] decolorization assay, as described elsewhere [33]. Antioxidant reducing capacity was expressed as μM Trolox equivalents. Phenolic content in the diet samples was determined using the Folin-Ciocalteu method [34,35].

Statistical Analysis

Results are expressed as mean ± SEM. Significance of differences between a diet intervention group (WO or WW group) and the control group (HFC) was determined with a parametric t test or a non-parametric Mann–Whitney U test, as required, using Prism (GraphPad Software version 5.00, San Diego CA, USA). Statistical significance was set at $p < 0.05$.

## Results

Dietary Intervention with Walnuts or Walnut Oil

Three groups of 12 $Apoe^{-/-}$ mice were fed with high fat diets (33–34 energy% fat, 0.2 weight% cholesterol), with compositions as shown in the Supplementary Table 1. The control (HFC) group received a diet enriched in sunflower oil with n-6 PUFA (66%). In the walnut oil (WO) group, the sunflower oil was replaced by purified walnut oil, resulting in a high content of n-3 PUFA (10%). In the whole walnut (WW) group, complete walnuts were given, rich in both n-3 PUFA (13%) and other walnut components. Energy, carbohydrate, protein, fat, as well as fiber and cholesterol contents were similar in all diets. In addition, fractions of saturated fatty acids (9-11%) were always low. Analysis indicated that the WW diet was five-fold enriched in water-soluble antioxidants and had a 50% higher content of phenolic compounds, compared to the WO an HFC diets (Supplementary Table 1). During the dietary period of 8 weeks, mice of all groups similarly increased in body weight, starting from 23–24 g and reaching 36–38 g (Supplementary Fig. 1).

Effects of Walnut Diets on Lipid Profiles

After the 8 weeks of diet, blood plasma and livers from all mice were analyzed for lipid content. In the WW group, plasma levels of triglycerides and total cholesterol were reduced with 36% and 23%, respectively, in comparison to the HFC group (Fig. 1A, B). In contrast, plasma triglycerides and cholesterol of the WO group were comparable to the control group. Profiles of cholesterol in lipoproteins were similar after all diets (Fig. 1C), thus pointing to an overall reduction in cholesterol of most lipoprotein classes (except HDL) in the WW group. In a separate control group, plasma triglycerides and cholesterol levels were measured as baseline values at 0.64 ± 0.24 mM and 19.90 ± 3.7 mM, respectively (mean ± sd, $n = 10$). Liver sections showed the presence of relatively small lipid droplets in only the WW group in comparison to the HFC group (Fig. 1D). Liver triglycerides were reduced by 30% in the WW group, whereas no significant reduction was observed in the WO group (Fig. 1E).

Effect of Walnut Diets on Atherosclerotic Plaque Development

Aortic arches including main branch points from all mice were isolated and sectioned to determine the development of atherosclerotic plaques in the aortic region. Per arch, four sections were analyzed for the determination of lesion size and the expression of CD36, as a marker of atherosclerosis. Remarkably, animals of the WW group showed a 55% reduction in overall atherosclerotic plaque area, compared to the HFC group (Fig. 2A-C). In contrast, plaque size in animals of the WO group was similar to the control group. Immunohistochemistry indicated high staining for the scavenger receptor CD36 within plaque areas, at sites where (CD36-expressing) macrophages were present (Supplementary Fig. 2). Staining of CD36 was about 50% lower in the WW group compared to the HFC group (Fig. 2D). Again, no difference was observed between the WO and HFC groups. For all groups together, staining for CD36 correlated significantly with plaque area ($R^2 = 0.72$, $p = 0.0013$). Together, these data indicated that dietary supplementation with walnuts, but not with walnut oil, restrained the formation of CD36-expressing atherosclerotic plaques in the aortic region of $Apoe^{-/-}$ mice on high fat diet.
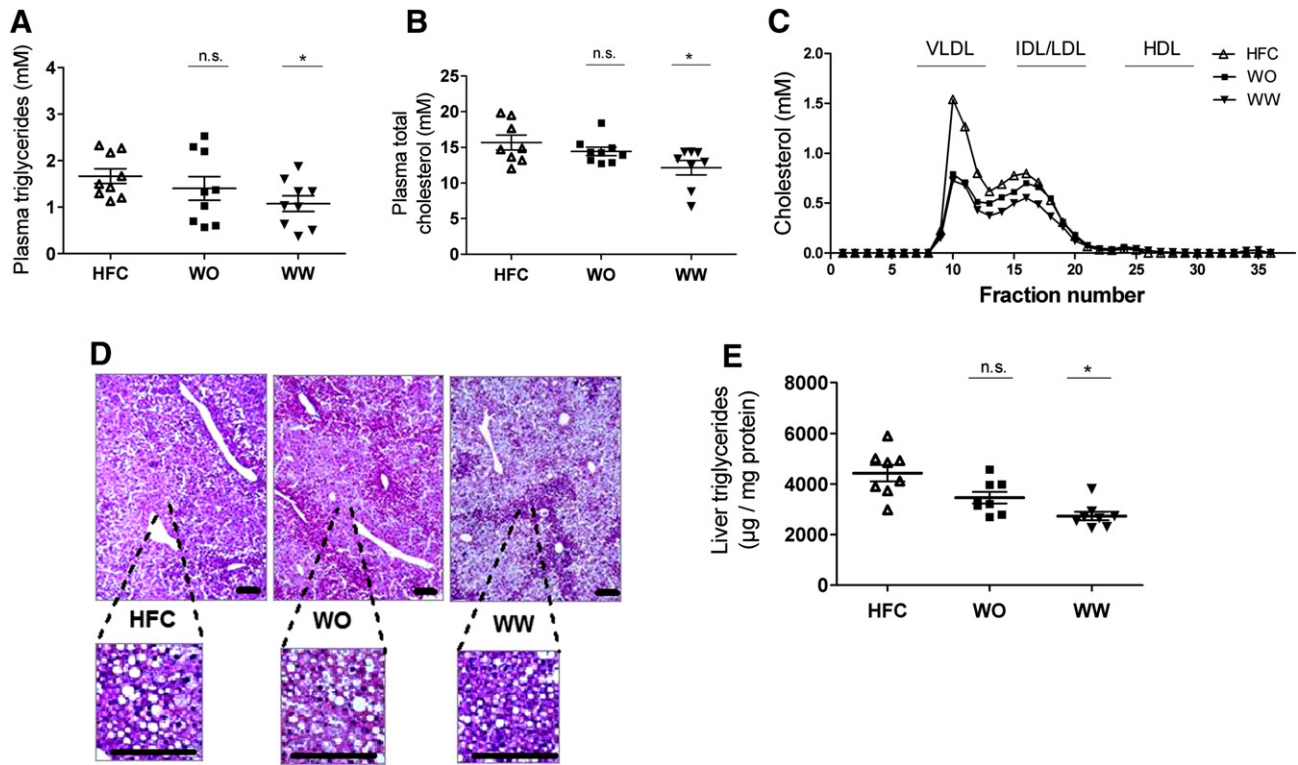
Fig. 1. Walnut diet lowers plasma lipids and diminishes lipid accumulation in livers from $Apoe^{-/-}$ mice. Plasma levels of (A) triglycerides and (B) total cholesterol in mice of the HFC, WO and WW groups. (C) Plasma cholesterol levels in different lipoprotein fractions. (D) Representative paraffin sections of mouse livers stained with hematoxylin-eosin. Bars = 100 μm, with magnified images showing fat droplets. (E) Triglyceride levels in mouse livers for all diet groups. Means ± S.E.M ($n = 8$); *$p < 0.05$ vs. HFC group; n.s., not significant.

Effects of Walnut Diets on Coagulation Factors and Antioxidant Capacity

Considering evidence that dietary *n*-3 PUFA in fish oil reduce plasma lipids and coagulant activity in hyperlipidemic patients and mice [8,9], we also measured these parameters in the present study. Plasma level of prothrombin was reduced by 21% in the WW group in comparison to the HFC group, but levels of fibrinogen or factor V were not different (Fig. 3A-C). For all groups together, plasma levels

of prothrombin and factor V were closely correlated ($R^2 = 0.67$, $p = 0.02$). As an overall test of the plasma coagulant activity, thrombin generation was measured in tissue factor-triggered plasma [36]. Thrombin peak height - *i.e.* a measure of the maximal rate of thrombin generation - was not different between the groups, although it tended to be lower in the WW group (Fig. 3D).

Plasma samples were furthermore analyzed for Trolox equivalent antioxidant capacity (TEAC). The higher intake of water-soluble
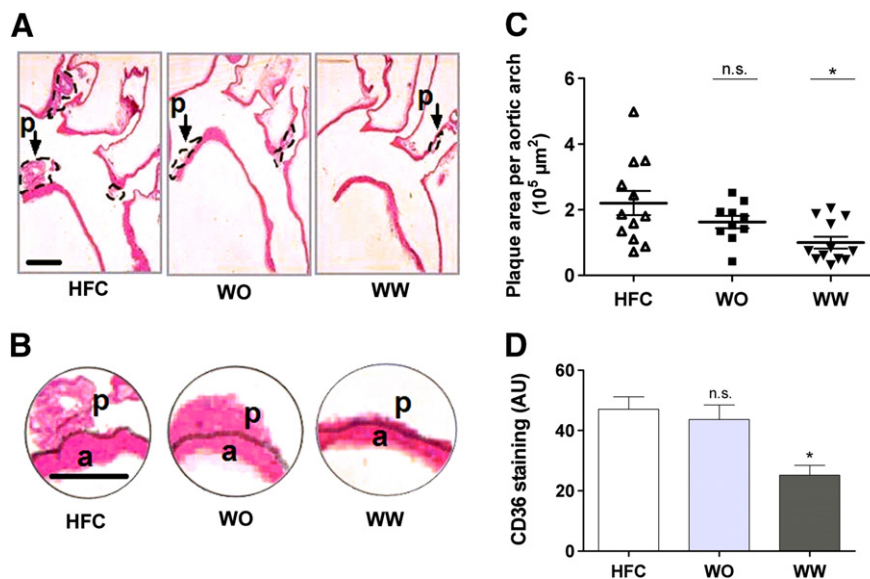
Fig. 2. Whole walnut diet reduces atherosclerotic lesion development in $Apoe^{-/-}$ mice. Progression of atherosclerotic plaques was analyzed in aortic arches from all mice after 8 weeks of HFC, WO or WW diet. (A) Representative HE-stained sections with plaques (bars, 1 mm). (B) Magnified areas indicating aortic wall (a) and plaque (p). (C) Plaque content in aortic arches; data points representing values from individual mice (average of 4 sections per aorta). (D) Quantification of CD36 staining intensity of identified plaque areas. Means ± SEM ($n = 10$-12); *$p < 0.05$ vs. HFC group; n.s., not significant.

antioxidants in the WW group was reflected in a significantly increased antioxidant capacity in WW plasmas, but not WO plasmas, when compared to the HFC group (Supplementary Fig. 3).

Effect of Walnut Diets on Platelet Activation and Thrombus Formation

Knowing that *in vitro* addition of antioxidants can inhibit blood platelet function [38,39], we determined the effects of the WO and WW diets on platelet activation markers. Flow cytometry showed that $\alpha_{IIb}\beta_3$ integrin activation (measured with JON/A mAb) and α-granule secretion (measured with anti-CD62P mAb) were unchanged in response to the key agonists ADP, convulxin or PAR4 peptide, when the WW and WO groups were compared to the HFC group (Fig. 4A, B). Platelet reactivity was also measured under more physiological conditions, i.e. in whole blood perfused over a collagen surface at arterial shear rate [30]. In this *ex vivo* assay, platelets from all diet groups were similarly active in forming aggregates (Fig. 4C, D). Post-staining of the platelets with FITC-annexin A5 to probe procoagulant activity also was unchanged in the WW and WO groups. Accordingly, no dietary effect of WW or WO in comparison to HFC, could be detected on platelet function.

Discussion

In this paper, we investigated the effects of walnuts and walnut oil on markers of atherothrombosis in $Apoe^{-/-}$ mice, which were given to the animals as part of a high fat and cholesterol containing diet, in comparison to a high fat sunflower oil diet. We found that only the intake of whole walnuts, but not of walnut oil, caused a major reduction in atherosclerotic plaque formation. This diet effect was accompanied by a lowering of the triglycerides in blood plasma and the liver, an increased anticoagulant capacity of plasma, and a slight reduction in plasma prothrombin, but not by detectable changes in platelet activation or coagulation activation. In contrast, the walnut oil diet did not change these parameters in comparison to the sunflower oil diet.

Walnuts are particularly interesting as dietary components, since they contain both antioxidant polyphenols and *n*-3 PUFA (mainly α-linolenic acid), both of which are considered to promote cardiovascular health. In the present study, we ascertained that the contents of water-soluble antioxidants as well as phenolics were enriched in WW diet, in comparison to the WO diet, whereas both diets were similarly enriched in *n*-3 PUFA. Since intake of walnut oil did not mimic the atheroprotective and lipid-lowering effects of whole walnut intake, it is unlikely that these changes were due to the intake of *n*-3 PUFA alone. In apparent contrast, in an earlier study with APOE2 knock-in mice, we found that fish oil intake resulted in lowered plasma lipids and reduced coagulant activity [9]. However, given the much higher levels of *n*-3 PUFA in fish oil (59% eicosapentaenoic plus docosahexaenoic acids) than in walnut oil (14% α-linolenic acid), it is conceivable that in the present study the amount of *n*-3 PUFA intake was too low for these changes to occur.

The ability of walnuts, but not walnut oil, to greatly restrain atherosclerotic plaque progression in comparison to a high fat control diet with *n*-6 PUFA is a novel finding. An earlier, small study pointed to somewhat increased lipid levels and increased atherosclerosis in $Apoe^{-/-}$ mice fed with walnut oil [40]. On the other hand, data from a human intervention study indicated that the regular intake of walnuts associates with a lowering of plasma triglycerides [41]. Compatible with the present results, several reports indicate that walnut extracts in cultured cells reduce the expression of cell adhesion molecules and lower the release of proinflammatory cytokines [42–44].

Interestingly, we measure a reduced expression of CD36 in the plaque regions of the aortic arch after walnut intake, which is a relevant finding, given that CD36 is mainly localized on macrophages in the atherosclerotic lesions [45,46]. It has also been shown that CD36 contributes to the atherosclerotic process, since CD36 deficiency in $Apoe^{-/-}$ mice protects against plaque development [47,48].

Collectively, the data of this mouse study do not point to more than minor effects of walnut or walnut-oil intake on platelet and coagulation activities. After walnut intake, prothrombin in plasma was moderately lowered, while fibrinogen and factor V levels remained unaltered. Similarly, human intervention studies do not report on effects of walnut intake on coagulation parameters [49,50] or platelet activation [18]. We have to note that smaller numbers of animals were used for the platelet activation markers and thrombus formation compared to the plasma markers for cholesterol, triglycerides and coagulation factors. This may be a limitation of the study.

It is tentative to assume that the atheroprotective effect of walnuts is a consequence of the simultaneous presence of α-linolenic acid and
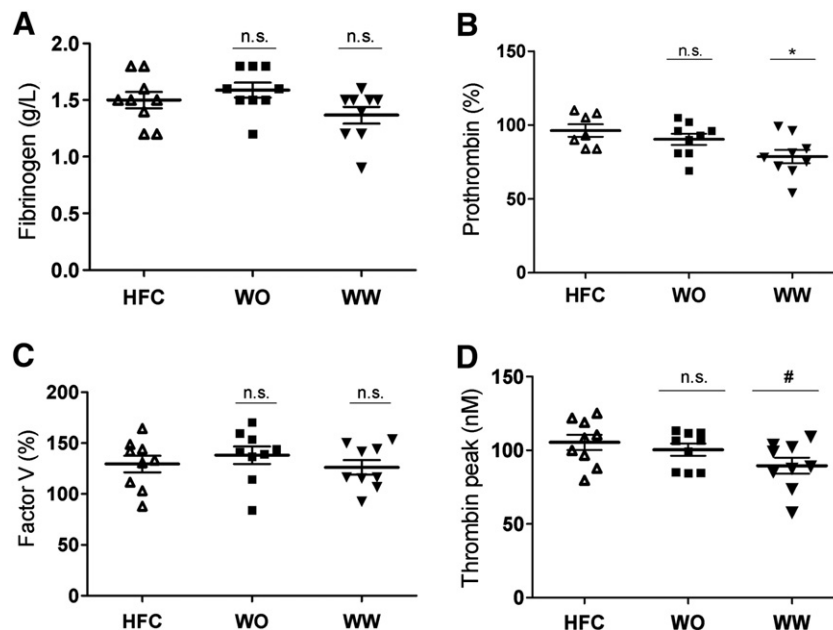
Fig. 3. Moderate effect of whole walnut diet on coagulation parameters. Plasma samples of mice from all diet groups were analyzed for coagulation factors and thrombin generation potential, using the calibrated automated thrombin generation assay. Shown are plasma levels of (A) fibrinogen; (B) prothrombin; (C) factor V; (D) thrombin peak heights of thrombin generation curves. Means ± SEM ($n = 9$); *$p < 0.05$, # $p = 0.05$ *vs.* HFC group; n.s., not significant.
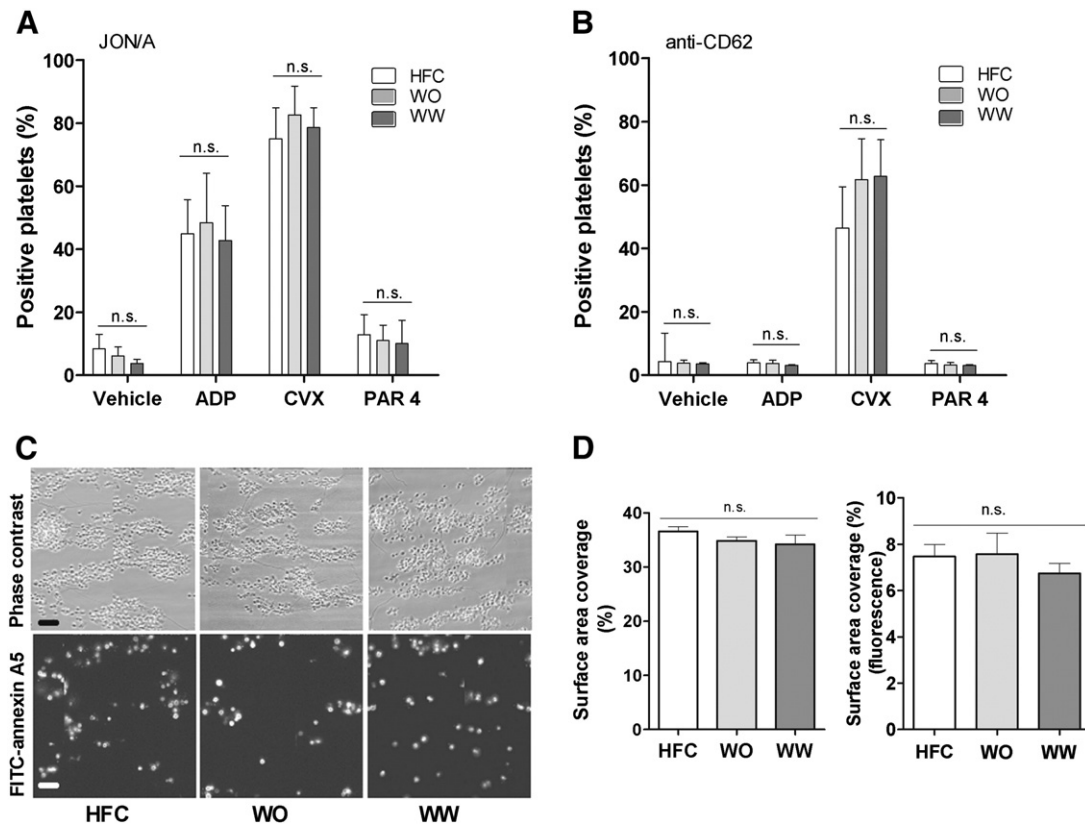
Fig. 4. No effect of walnut diets on platelet activation and thrombus formation. Blood from $Apoe^{-/-}$ mice, fed the various diets, was analyzed for platelet function and thrombus formation. (A, B) Isolated platelets were stimulated with ADP (40 μM), convulxin (CVX, 100 ng/ml) or PAR4 peptide GYPGKF (0.5 mM) for 10 min. (A) Activation of $\alpha_{IIb}\beta_3$ (PE-labeled JON/A mAb, 150 μg/ml) and (B) expression of P-selectin (FITC-anti-CD62P mAb, 1:50) were assessed by flow cytometry. (C, D) PPACK/heparin-anticoagulated whole blood was flowed 4 min over a collagen surface at 1000 s^{-1}. Thrombus formation was determined by analysis of platelet deposition and procoagulant platelet staining with FITC-labeled annexin A5. (C) Representative phase contrast and fluorescence images (bars, 10 μm). (D) Surface area coverage of thrombi and of fluorescently labeled procoagulant platelets. Data are means ± SEM (HFC and WW $n = 6$, WO $n = 5$); n.s., not significant compared to HFC group.

water-soluble antioxidant substances, including polyphenols. The literature indeed shows high bio-availability of the antioxidants in walnuts [51], and increased levels of TEAC in plasma after walnut consumption [37]. The antioxidant polyphenolics of walnuts (estimated 16–18 mg/g) appear to be a complex mixture, which has only partly been characterized with as main constituents, ellagic acid, ellagitannins, gallic acid and hydrocinnamic acids like chlorogenic acid [52,53]. Likely, these components contribute to the increased plasma antioxidant capacity, seen in the present study. However, the precise contribution of antioxidant polyphenols still needs to be determined.

Taken together, we report a beneficial effect of dietary walnut intake on atherosclerotic plaque development and plasma lipids in hyperlipidemic $Apoe^{-/-}$ mice, with no more than limited effects on platelet and coagulation function. Furthermore, we find that the atheroprotective effect of walnut involves other components than merely n-3 PUFA.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2013.01.003.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Acknowledgements

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