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# Nanoencapsulation of the omega-3 EPA:DHA 6:1 formulation enhances and sustains NO-mediated endothelium-dependent relaxations in coronary artery rings and NO formation in endothelial cells

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ABSTRACT

The eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) formulation with a ratio of 6:1 is a potent stimulator of the endothelial formation of nitric oxide (NO). The aim of the study was to investigate whether nanoencapsulation of EPA:DHA 6:1 followed by coating with gum increases its biological activity. Vascular reactivity was assessed using porcine coronary artery rings, the formation of NO in cultured endothelial cells (ECs) using DAF-FM and indirectly by platelet aggregation studies. Coated EPA:DHA 6:1 nanoparticles induced sustained relaxations of coronary artery rings that were greater in rings with than in those without endothelium, and more pronounced than with the native form. Treatment of ECs with coated EPA:DHA 6:1 nanoparticles caused greater and more sustained formation of NO and enhanced their anti-aggregatory effects. Thus, nanoencapsulation of EPA:DHA 6:1 is an attractive strategy to enhance the beneficial effect at the vascular endothelium.

## 1. Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality worldwide and account for approximately one-third of all deaths (Mozaffarian et al., 2015). The prevalence of CVD including coronary heart disease, heart failure, and stroke increases with age in both man and women in particular after 40 years of age (Benjamin et al., 2018). Given the heavy burden CVD present for health and economy, prevention and management of CVD is a major target of ongoing research and clinical practice (Arnett et al., 2019).

Endothelial cells (EC) play a pivotal role in the control of vascular homeostasis mostly due to several vasoprotective mechanisms including nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) (Siasos, 2020). Endothelial dysfunction, a major early hallmark of CVD, is characterized by a reduced formation of nitric oxide (NO) and EDH and, often also, the appearance of endothelium-dependent contractile responses (EDCFs) involving vasoconstrictor prostanoids generated by the cyclooxygenase (COX) pathway (Siasos et al., 2015; Vanhoutte et al., 2009). Since endothelial dysfunction promotes excessive vasoconstriction, arterial remodeling, and atherothrombotic responses (Siasos et al., 2015), the regeneration of EC is a challenging therapeutic strategy to protect the cardiovascular system.

Several clinical and animal studies have shown that the consumption of omega-3 polyunsaturated fatty acids (PUFAs), including the two main compounds eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is associated with a beneficial effect on the cardiovascular system (DiNicolantonio et al., 2014; Iwamatsu et al., 2016; Kromhout et al., 2012). In 1980, Bang et al. (Bang et al., 1976) observed an inverse relation between the intake of a diet rich in long chain PUFAs and the incidence of CVDs among the Eskimo population of Greenland (Bjerregaard, 1991). Similarly, clinical trials such as DART (1989), GISSIPrevenzione (1999), GISSI-HF (2004) and JELIS (2007) showed a reduction of the risk of cardiovascular deaths with daily consumption of omega-3 PUFAs (Bowen et al., 2016). More recently, the REDUCE-IT trial including patients with previously established cardiovascular diseases and a high level of triglycerides showed a 25% reduction of major cardiovascular events after ingestion of 4 g/day of icosapent ethyl EPA along with statin therapy (Bhatt, Steg, Miller, Brinton, Jacobson,

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Ketchum, Doyle, Juliano, Jiao, & Granowitz, 2019; Bhatt, Steg, Miller, Brinton, Jacobson, Jiao, Tardif, Gregson, Pocock, & Ballantyne, 2019; Bhatt, Steg, Miller, Brinton, Jacobson, Ketchum, Doyle, Juliano, Jiao, Granowitz, Tardif, Ballantyne, & Investigators, 2019; Gajos, 2019). The cardiovascular beneficial effect of omega-3 PUFAs has been attributable to their ability to reduce the level of triglycerides, platelet activation, pro-inflammatory responses, blood pressure, and also the improvement of the protective endothelial function. Of importance, the vasoprotective effect of omega-3 PUFAs is dependent on both the ratio and the purity of EPA and DHA preparations (Zgheel et al., 2014). Among several omega-3 formulations, EPA:DHA 6:1 caused pronounced NO-dependent relaxation of porcine coronary artery rings subsequent to the endothelial NO synthase (eNOS) activation via the Src/PI3-kinase/Akt and MAPKs pathways (Zgheel et al., 2014). In addition, EPA:DHA 6:1 reduced systolic blood pressure in angiotensin II-induced hypertensive rats and improved endothelial dysfunction and vascular oxidative stress (Niazi et al., 2017), and also improved established ageing-related endothelial dysfunction by targeting the AT1R/NADPH oxidase pathway (Farooq et al., 2020a, 2020b). Moreover, this formulation effectively prevented platelet-induced and serotonin-mediated contractile responses in porcine coronary artery and human internal mammary artery rings (Zgheel et al., 2019).

The aim of the study was to investigate whether nanoencapsulation of EPA:DHA 6:1 potentiates their beneficial effect on the endothelial function as assessed using isolated artery rings and cultured endothelial cells.

## 2. Materials and methods

### 2.1. Omega-3 PUFAs formulations and corn oil

The omega-3 EPA:DHA 6:1 (w/w) formulation was similar as that previously characterized and studied by the group (Zgheel et al., 2014). It was kindly provided by Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada). The coated EPA:DHA 6:1 nanoemulsion was prepared by emulsification of omega-3 fatty acids into a surfactant solution containing Tween 80, Span 80 and lecithin. The emulsion is further mixed with a gelatin and gummi arabicum solution. pH of the final emulsion was adjusted to 4.8 with 10% acetic acid. Corn oil (Mazola) contained saturated fatty acids (12 g), palmitic acid (11 g), stearic acid (1 g), monosaturated fatty acids (28 g), oleic acid (28 g), polyunsaturated fatty acid (52 g), and linoleic acid (52 g).

### 2.2. Vascular reactivity studies

Vascular reactivity studies were performed using the left circumflex coronary artery of pig hearts collected from the local slaughterhouse (SOCOPA, Holtzheim, France). They were excised, cleaned of conjunctive tissues and cut into rings (4–5 mm in length). In some rings, the endothelium was removed mechanically by gently rubbing the lumen of the ring with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% $O_2$, 5% $CO_2$) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37 °C). The resting tension was set at 5 g before the assessment of changes in isometric tension. After the equilibration period, rings were exposed to a high $K^+$-containing Krebs bicarbonate solution (80 mM) until reproducible contractile responses were obtained. To assess the endothelial function, the rings were contracted with U46619 (10 nM) before the induction of a relaxation to bradykinin (0.3 μM). After washing and a 30-min resting period, coronary artery rings were contracted again with U46619 (10 nM) before assessment of the relaxation to either native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles in rings both with and without endothelium. Since preliminary investigations have shown that the development of an endothelial-dependent relaxation is slower and more sustained with the coated nanoformulation than with the native one, the relaxation was evaluated in response to a single concentration of an omega-3 formulation and over a 60-min period. In some experiments, rings were incubated with a modulator for 30 min before the addition of U46619, and the subsequent relaxation to either native or coated EPA:DHA 6:1 nanoparticles.

### 2.3. Primary coronary artery endothelial cell culture

Left circumflex coronary arteries were excised, cleaned and flushed with PBS without calcium to remove remaining blood. Coronary arteries were treated with 1 mg/ml of collagenase type I (ThermoFisher) for 15 min at 37 °C. Thereafter, ECs were collected and cultured in T25 flasks containing MCDB 131 (Invitrogen, LifeTechnologies SAS, Courtaboeuf, France) medium supplemented with fungizone (2.5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM, all from Lonza, Levallois-Perret, France) and 15% fetal bovine serum. Thereafter, ECs were grown to 80–90% confluence over a 48–72-h period. Experiments were performed with cultured ECs at passage 1, which were exposed to serum-free culture medium for 2 h before the addition of either bradykinin or EPA:DHA 6:1.

### 2.4. Detection of nitric oxide (NO) formation

ECs were cultured on 8-wells Lab-Tek® chambers and were exposed to serum-free culture medium for 2 h before the addition of bradykinin (100 nM) or native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles for 24 h. In some experiments, ECs were treated with either bradykinin (100 nM, 24 h) or $N^{\omega}$-nitro-L-arginine (an inhibitor of NOS, L-NA, 300 μM, 30 min) before the addition of either native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles for 24 h. Thereafter, ECs were exposed to DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluororescein diacetate, 1 μM, a NO-sensitive fluorescent dye) for 20 min at 37 °C in the dark, followed by washing with PBS. After disassembling, slides were mounted with fluorescence mounting medium (Agilent Technologies France, Les Ulis, France) and cover-slipped before being analyzed using confocal laser-scanning microscope (Leica SP2 UV DM IRBE, Heidelberg, Germany) with a 63X magnification lens. Quantification of fluorescence levels was performed using Image J software (version 1.49 for Windows, NIH).

### 2.5. Determination of platelet aggregation

Washed human platelets were provided by the Etablissement Français du Sang - Alsace (Strasbourg), and suspended in Tyrode buffer at $3.10^8$ platelets/ml. Suspensions of washed platelets (450 μl) were incubated with continuous stirring (1000 r.p.m, 37 °C) in an aggregometer (Chronolog 490, Diagnostica Stago SAS, Asnière sur Seine, France). ECs were cultured on Cytodex-3 microcarrier beads, which were hydrated and sterilized according to the instructions supplied by the manufacturer (GE Healthcare Life Sciences, Strasbourg, France). ECs at passage 1 grown on Cytodex-3 beads were exposed to serum-free culture medium for 2 h before the treatment with either native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles for 24 h. A volume of 10-35 μl of beads covered with ECs (about 2000 cells/ml) was added to suspensions of platelets for 1 min before the addition of thrombin (0.025 U/ml) to induce platelet aggregation. In some experiments, treated ECs with coated EPA:DHA 6:1 nanoparticles were incubated with L-NA (300 μM) for 30 min before the addition to the platelet suspension.

### 2.6. Statistical analysis

Results are presented as means ± S.E.M for n different experiments and analyzed by Graphpad Prism (Version 7). Mean values were compared between different groups by using Two-way or One-way ANOVA test followed by Bonferroni's Multiple Comparison *post hoc* test. Group differences were considered statistically significant at $P <$

0.05.

## 3. Results

The nanoemulsion was characterized for particle size and furthermore stability evaluation was conducted under accelerated conditions. The initial particle size of the nanoemulsion was 273.6 nm with a polydispersity index (PDI) of 0.182. There was no significant change in particle size after a 1-month storage period at 40 °C (accelerated stability screening condition). The results revealed a particle size of 275.2 nm and a PDI of 0.175 at the end of 1 month.

### *3.1. Coated EPA:DHA 6:1 nanoparticles cause sustained endothelium-dependent relaxations of coronary artery rings than the native form*

To study the vasorelaxant effect of omega-3 EPA:DHA 6:1 formulations, porcine coronary artery rings with and without endothelium were contracted with U46619 before the addition of an omega-3 PUFAs formulation. Time course studies indicated that the relaxation to the native form from 0.1 to 0.4% v/v reached a maximal value after about 10 min and, thereafter, declined progressively towards baseline (Fig. 1A-D). In contrast, relaxations to the coated EPA:DHA 6:1 nanoparticles increased progressively to reach a near maximal value at 60 min (Fig. 1A-D). Relaxations to both native and coated EPA:DHA 6:1 nanoparticles were more pronounced in rings with than in those without endothelium (Fig. 1A-D). Although relaxations at 10 min were similar in response to the native and the coated formulation, those at 60 min were markedly greater with the coated formulation at 0.1, 0.2, 0.3 and 0.4% v/v (the values were 39.7 ± 2.2 and 95.3 ± 1.2 for native and coated EPA:DHA 6:1 formulations at 0.4% v/v, respectively; Fig. 1D,E,F).

### *3.2. The sustained endothelium-dependent relaxation to coated EPA:DHA 6:1 nanoparticles involves predominantly NO*

Next, experiments were performed to determine the role of the different endothelium-derived relaxing factors in the relaxation induced by both EPA:DHA 6:1 formulations. The endothelium-dependent relaxation to coated EPA:DHA 6:1 nanoparticles at 0.3% v/v is abolished by $N^{\omega}$-nitro-L-arginine (L-NA, a non-selective NOS inhibitor) and affected neither by indomethacin (a non-selective cyclooxygenase inhibitor) nor

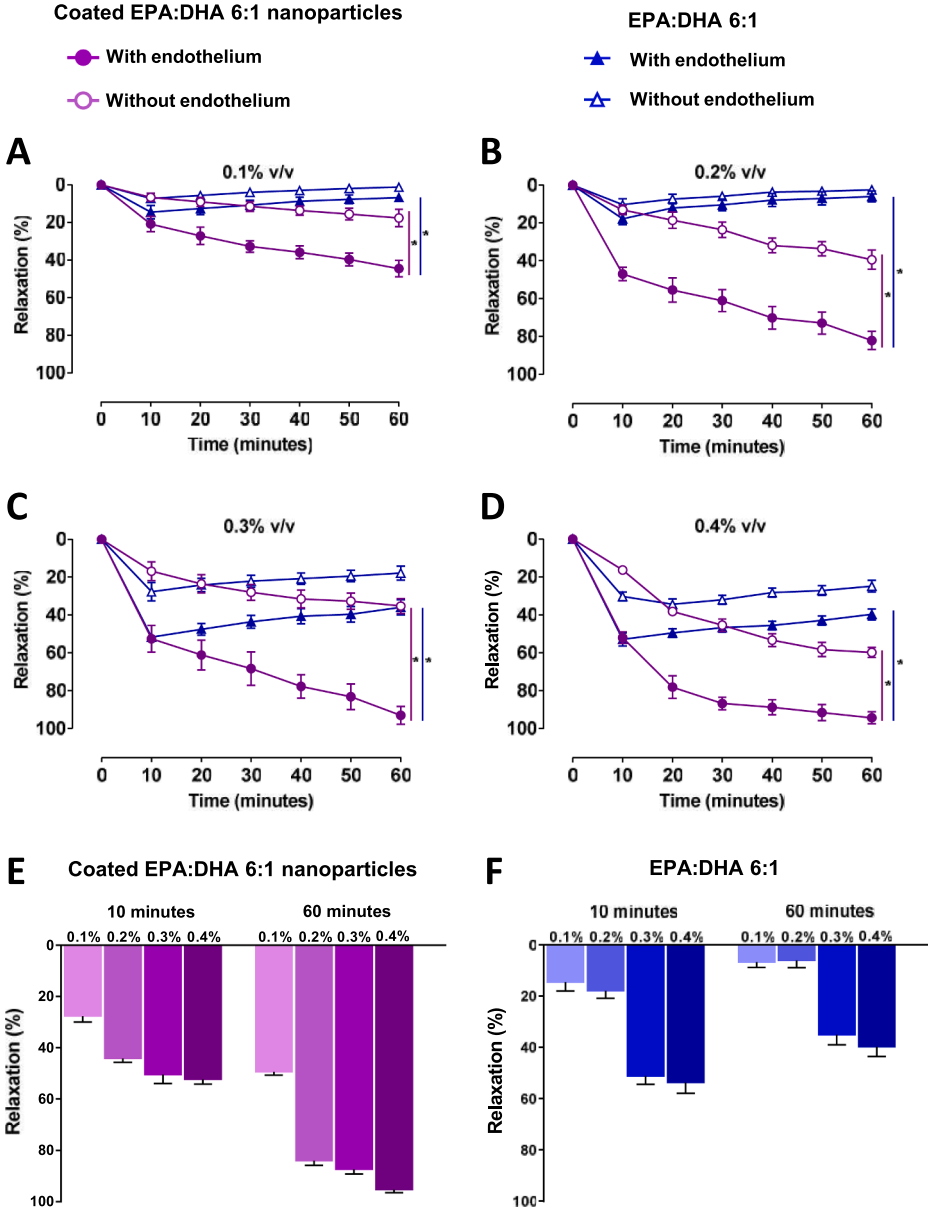


**Fig. 1. Coated EPA:DHA 6:1 nanoparticles promote greater and sustained endothelium-dependent relaxations than the native form in coronary artery rings.** Coronary artery rings with or without endothelium were contracted with U46619 (10 nM) before the addition of either coated EPA:DHA 6:1 nanoparticles or native EPA:DHA 6:1 form and assessment of the relaxation over a 60-min period. Results are expressed in% relaxations as means ± SEM of 4–6 different experiments. * $P <$ 0.05 vs. Control (Coated EPA:DHA 6:1 nanoparticles with endothelium).

by the inhibition of EDH responses using the combination of TRAM-34 plus UCL-1684 (inhibitors of $IK_{Ca}$ and $SK_{Ca}$, respectively) indicating the exclusive involvement of NO (Fig. 2A,C). In contrast, the relaxation to the native EPA:DHA 6:1 form was inhibited significantly to some extent by indomethacin in both rings with and without endothelium indicating the involvement of vasorelaxant prostanoids (Fig. 2B). In addition to omega-3 PUFAs, experiments have also assessed the vasoactive effect of an isocaloric oil, corn oil, in rings with and without endothelium. The addition of corn oil to precontracted rings did affect neither vascular tone and nor the subsequent relaxation induced by coated EPA:DHA 6:1 nanoparticles at 0.3% v/v in rings with endothelium (Fig. 2C,D). In rings without endothelium, the relaxation to coated EPA:DHA 6:1 nanoparticles at 0.3% v/v was not affected by corn oil during the first 20 min and thereafter was slightly but significantly reduced (Fig. 2D).

### 3.3. Coated EPA:DHA 6:1 nanoparticles induce a sustained formation of NO in cultured endothelial cells

Previously, we have shown that EPA:DHA 6:1 is able to activate the PI3-kinase/Akt/eNOS pathway within 30 min in cultured coronary artery endothelial cells (Zgheel et al., 2014). Since a sustained endothelium-dependent relaxation was observed in response to coated EPA:DHA 6:1 nanoparticles, their ability to cause the formation of NO in coronary artery endothelial cells over a 24-h period was assessed using DAF-FM, a fluorescence NO indicator, and compared to that induced by the native omega-3 formulation and the physiological agonist bradykinin. Coated EPA:DHA 6:1 nanoparticles increased the DAF-related fluorescence starting at a concentration of 0.001% v/v and reached about 2.5-fold at 0.003% v/v at 24 h whereas the native formulation increased the DAF signal only at 0.01% v/v (Fig. 3). In contrast, a small stimulatory effect was observed with bradykinin after 24 h, which amounted to about 1.5-fold (Fig. 3). The stimulatory effect of both coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 at 0.3% v/v after 24 h was abolished by L-NA demonstrating the involvement of NO (Fig. 4). L-NA also reduced the small stimulatory effect of bradykinin at 24 h, which, however, did not reach statistical significance (Fig. 4).

### 3.4. Coated EPA:DHA 6:1 nanoparticles potentiate the anti-aggregatory effect of ECs

Next, the potential of the omega-3 formulations to potentiate the antiaggregatory effect of ECs was determined by adding ECs cultured on Cytodex beads to suspensions of washed human platelets.

The addition of about 2000 ECs/ml to suspensions of platelets reduced by about 20% the sub-maximal aggregation induced by

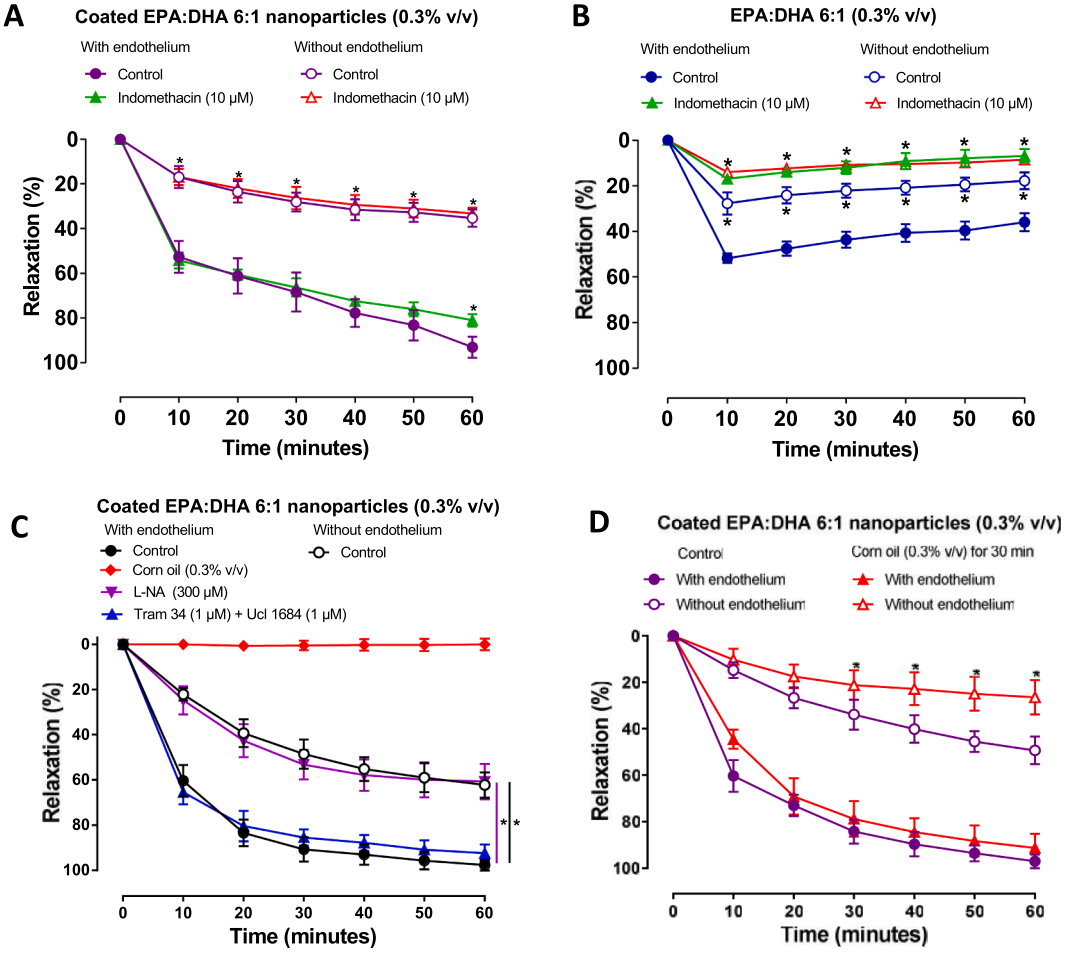


**Fig. 2. The coated EPA:DHA 6:1 nanoparticles-induced endothelium-dependent relaxation in porcine coronary artery rings involves predominantly NO and is not affected by corn oil.** Coronary artery rings with or without endothelium were contracted with U46619 (10 nM) before the addition of either coated EPA:DHA 6:1 nanoparticles or native EPA:DHA 6:1 form. The role of prostanoids was assessed using indomethacin (10 µM, a non-selective COX inhibitor, A), of NO using $N^{\omega}$-nitro-L-arginine (L-NA, 300 µM, a NOS inhibitor), and of endothelium-derived hyperpolarization using TRAM-34 and UCL-1684 (1 µM each, inhibitors of $IK_{Ca}$ and $SK_{Ca}$, respectively). (D) Coronary artery rings with and without endothelium were pre-incubated with corn oil (0.3% v/v) for 30 min before the addition of U46619, and the subsequent assessment of the relaxation to coated EPA:DHA 6:1 nanoparticles over a 60-min period. Results are expressed in% relaxations as means ± SEM of 5–6 different experiments. * $P < 0.05$ vs. Control without endothelium.

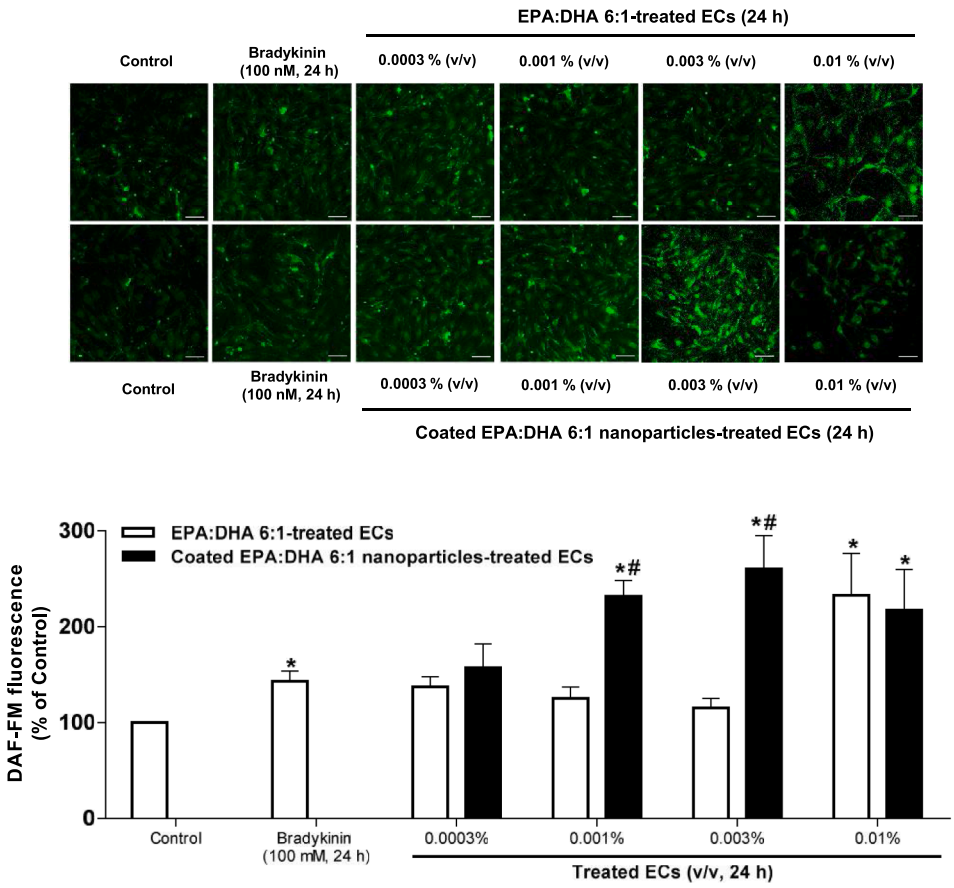


**Fig. 3. Coated EPA:DHA 6:1 nanoparticles induce greater and sustained NO formation in endothelial cells compared to the native form.** ECs were incubated with either bradykinin (100 nM), the native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles for 24 h. NO formation was assessed in ECs using the fluorescent probe DAF-FM (1 μM, a NO-sensitive fluorescent dye) and confocal laser-scanning microscope. Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as means ± SEM of 3–4 different experiments. $^{*}P < 0.05$ vs. Control. $^{\#}P < 0.05$ vs. Native EPA:DHA 6:1 form treated-ECs. Original magnification, 20x. Scale bar = 50 μm.

thrombin (0.025 U/ml, Fig. 5). In contrast, the thrombin-induced platelet aggregation was abolished in the presence of coated EPA:DHA 6:1 nanoparticles-treated ECs and little affected by native EPA:DHA 6:1-treated ECs (Fig. 5A). Moreover, pretreatment of coated EPA:DHA 6:1 nanoparticles-treated ECs with L-NA before their addition to the platelet suspension prevented the inhibitory effect demonstrating the involvement of NO (Fig. 5B).

## 4. Discussion

Circulating levels of omega-3 PUFAs have been inversely associated with the risk of cardiovascular diseases. The Framingham Heart Study Offspring cohort reported that, a low content of omega-3 PUFAs in erythrocyte membranes has been associated with an increased risk of cardiovascular and all-cause mortality (Harris et al., 2018). Several clinical studies and meta-analyses have evaluated the effects of various omega-3 PUFAs-rich products (fish, fish oil, krill oil and marine oil) or purified omega-3 PUFAs such as EPA and DHA in primary and secondary prevention of cardiovascular disease (Aung et al., 2018; Bhatt, Steg, Miller, Brinton, Jacobson, Ketchum, Doyle, Juliano, Jiao, & Granowitz, 2019; Bhatt, Steg, Miller, Brinton, Jacobson, Jiao, Tardif, Gregson, Pocock, & Ballantyne, 2019; Bhatt, Steg, Miller, Brinton, Jacobson, Ketchum, Doyle, Juliano, Jiao, Granowitz, Tardif, Ballantyne, & Investigators, 2019; Delgado-Lista, 2012; DiNicolantonio et al., 2014; Iwamatsu et al., 2016). Although some studies reported a beneficial effect of omega-3 PUFA supplementation on major cardiovascular endpoints (Delgado-Lista, 2012; Investigators, 1999; Maki et al., 2017), no such effect was observed in other studies (Aung et al., 2018; Enns et al., 2014). Such differences in outcomes might possibly relate to the use of different doses, omega-3 PUFA sources, the degree of purity, and the formulation of the omega-3 PUFA products (Kromhout, 2012). The beneficial effects of omega-3 PUFAs on the cardiovascular system have been attributable to several effects such as reduction of the chronic inflammatory response, inhibition of the thrombogenesis, reduction of hypertension, improvement of the myocardial function, and also an improvement of the pivotal protective endothelial function (Calvo et al., 2017; Mozaffarian & Wu, 2011). In the human body, PUFAs are prone to oxidation due to their exposure to free radicals and enzymes such as cyclooxygenases, lipoxygenases and cytochromes P450, and produce various reactive chemical species such as aldehydes and ketones that reduce their nutritional value and can exert adverse effects on human health. In addition, the lipid oxidation may induce oxidative stress and pro-inflammatory responses when the metabolites reach high concentrations (Tao, 2015). Therefore, the aim of the present study was to investigate whether nanoencapsulation of EPA:DHA 6:1 followed by coating of the microparticles with gum is able to enhance their beneficial effect of the endothelial function.

The present findings indicate that EPA:DHA 6:1 induces concentration-dependent relaxations in porcine coronary artery rings, which were more prominent in rings with endothelium as compared to rings without endothelium. These findings are consistent with previous ones showing endothelium-dependent relaxations to EPA:DHA 6:1 in porcine coronary artery rings (Zgheel et al., 2014), in primary mesenteric artery rings of old rats (Farooq et al., 2020a, 2020b), and in femoral artery rings of middle aged rats (Gaertner et al., 2020). Furthermore, the findings indicate that nanoencapsulation of EPA:DHA 6:1 followed by coating of the nanoparticles with gum resulted in a progressively developing relaxation reaching a near maximal response over 60 min whereas the relaxation of the native form was transient reaching a maximal value after 10 min, and, thereafter, declined progressively towards baseline. The coated EPA:DHA 6:1 nanoparticles-induced relaxation was more sustained than that induced by the native form indicating that the encapsulation process was associated with an enhanced biological activity. Although the underlying mechanisms still

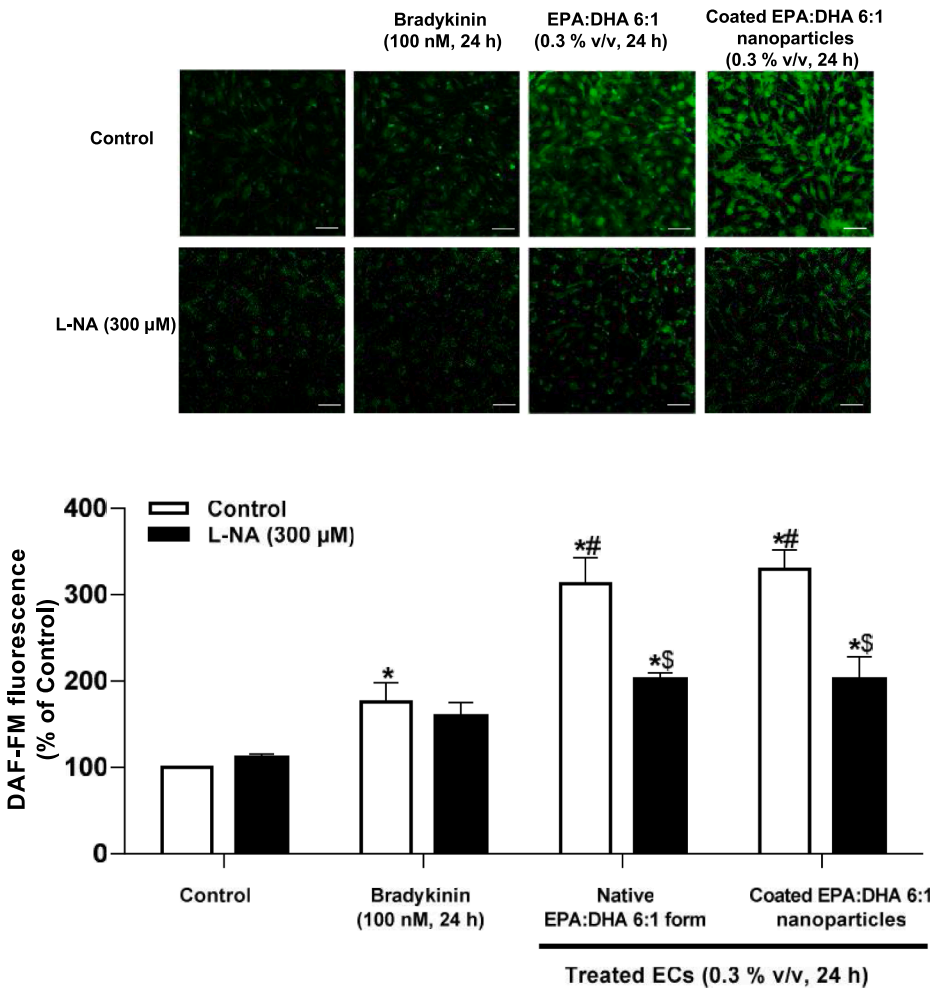


**Fig. 4. Native EPA:DHA 6:1 form and coated EPA:DHA 6:1 nanoparticles induce eNOS-derived NO formation in endothelial cells.** ECs were incubated with either bradykinin (100 nM, 24 h) or $N^{\omega}$-nitro-L-arginine (L-NA, 300 µM, 30 min) before the addition of either native EPA:DHA 6:1 form (0.3% v/v, 24 h) or coated EPA:DHA 6:1 nanoparticles (0.3% v/v, 24 h) . NO formation was assessed in ECs using the fluorescent probe DAF-FM (1 µM) and confocal laser-scanning microscope. Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as means ± SEM of n = 3. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Bradykinin. $^{\$}P < 0.05$ vs. respective Control. Original magnification, 20x. Scale bar = 50 µm.

remain to be clarified, they might include prolonged half-life of the omega-3 PUFAs, sustained interaction with the endothelial cell machinery and/or activation of the signal transduction cascade leading to eNOS activation (Tao, 2015).

The sustained endothelium-dependent relaxation to the coated EPA:DHA 6:1 nanoparticles was abolished by a non-selective NOS inhibitor (L-NA) and not affected by inhibition of either cyclooxygenases or EDH indicating the exclusive involvement of NO. In contrast, the relaxation in response to a single sub-maximal concentration of the native form was significantly reduced to some extent in rings with and in those without endothelium by indomethacin indicating the involvement of vasorelaxant prostanoids. In contrast, the previous study by the group observed that indomethacin did not affect concentration-relaxation curves to the native form in coronary artery rings (Zgheel et al., 2014). Such different responses observed with indomethacin are most likely due to the different experimental conditions (i.e., concentration-dependent relaxation curve versus relaxation to a single sub-maximal concentration, 15 min versus 60 min observation period).

The present findings are in agreement with our previous investigations showing that both the ratio and the purity of the EPA:DHA formulations are of major importance for the biological activity, as highly purified EPA:DHA 6:1 and 9:1 formulations demonstrated greater endothelium-dependent NO-mediated relaxation of porcine coronary artery rings than other ratios. Moreover, EPA:DHA 6:1 stimulated the redox-sensitive activation of Src/PI3-kinase/Akt and MAPK pathways leading to eNOS phosphorylation at Ser1177 and the subsequent formation of NO within 30 min in cultured coronary artery endothelial cells (Zgheel et al., 2014). EPA:DHA 6:1 also induced endothelium-dependent NO-mediated relaxations and inhibited serotonin-induced contractile responses in human internal thoracic artery rings (Zgheel et al., 2019). The present findings also indicate that in contrast to EPA:DHA 6:1, corn oil did induce neither endothelium-dependent relaxation nor affect the relaxation to coated EPA:DHA 6:1 nanoparticles. Since coated EPA:DHA 6:1 nanoparticles promoted long-lasting endothelium-dependent NO-mediated relaxations, experiments have assessed their ability to cause a sustained formation of NO in coronary artery ECs over a 24-h period. The findings indicate coated EPA:DHA 6:1 nanoparticles induced significantly higher endothelial levels of NO as compared with the native form whereas only low levels were observed with bradykinin over a 24-h period. In addition, the 24-h stimulatory effect of both the coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 was markedly reduced by L-NA indicating the involvement of NO. Thus, coated EPA:DHA 6:1 nanoparticles appears to be an interesting omega-3 PUFAs formulation promoting long-lasting endothelial formation of NO. In addition, since ECs-derived NO is a strong inhibitor of platelet aggregation (Khemais-Benkhiat et al., 2020), we investigated the anti-aggregatory effect of cultured ECs treated with either coated nano-encapsulated or native EPA:DHA 6:1 on human platelets. Although the addition of low numbers of EPA:DHA 6:1-treated ECs did not affect thrombin-induced aggregation, a pronounced inhibitory effect was observed in response to the addition of a similar number of coated EPA:DHA 6:1 nanoparticles-treated ECs. Since the inhibitory effect was abolished by L-NA, it implies the involvement of NO.

In conclusion, the major novel finding of this study is that nano-encapsulation of EPA:DHA 6:1 followed by coating is associated with an increased ability to stimulate the endothelial formation of NO leading to

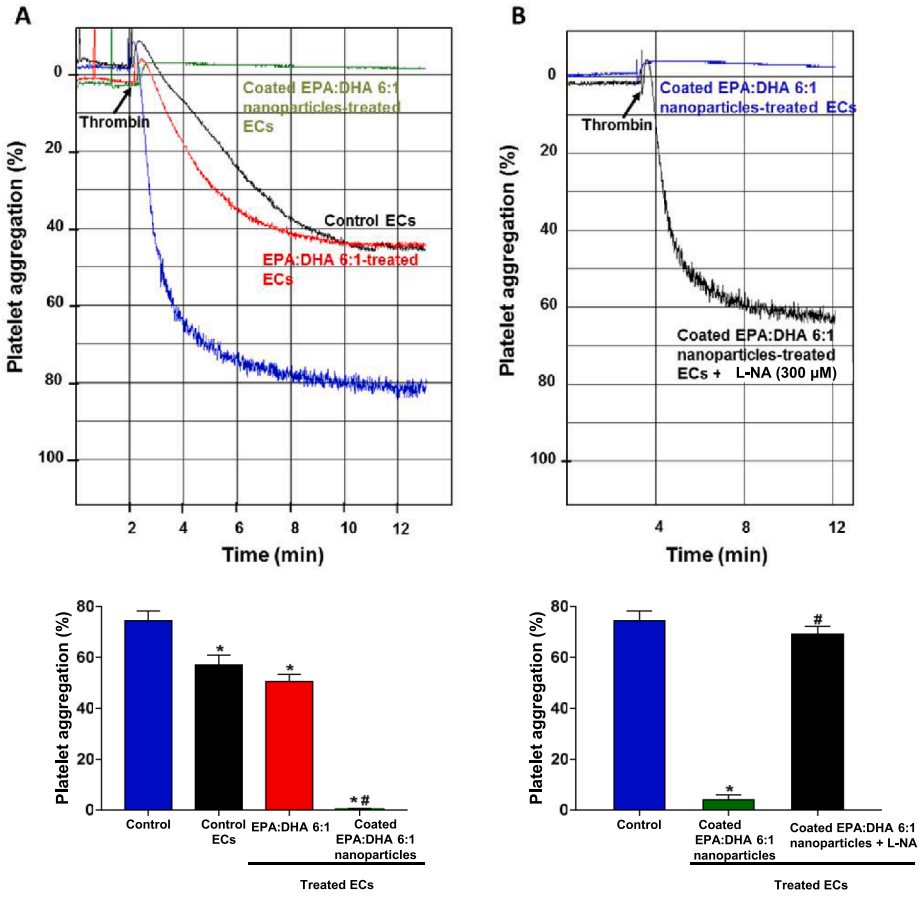


**Fig. 5. Coated EPA:DHA 6:1 nanoparticles-treated endothelial cells have a greater anti-aggregatory effect than those treated with the native omega-3 formulation.** ECs were cultured on Cytodex 3$^{TM}$ beads and treated with either native EPA:DHA 6:1 form (0.003%) or coated EPA:DHA 6:1 nanoparticles (0.003%) for 24 h. Thereafter, (A) untreated or treated ECs were added to suspensions of platelets for 1 min before the induction of platelet aggregation with thrombin (0.025 U/ml). (B) ECs treated with coated EPA:DHA 6:1 nanoparticles were incubated with L-NA (300 µM) for 30 min before the induction of platelet aggregation with thrombin. Representative platelet aggregation traces (upper panels) and corresponding quantitative analysis (lower panels). Results are expressed as mean ± SEM of 3–4 different experiments. $^{*}P < 0.05$ vs. Control (no cells), $^{\#}P < 0.05$ vs. Control ECs, $^{\#}P < 0.05$ vs. Coated EPA:DHA 6:1 nanoparticles-treated ECs.

a sustained and more prolonged endothelium-dependent vasorelaxation and inhibition of platelet aggregation. The results further suggest that coated omega-3 PUFAs nanoformulations appear as an interesting approach to better protect the endothelial function and, hence, the cardiovascular system.

## Ethical statement

The study doesn't involve human subjects and/or animals.

*CRediT authorship contribution statement*

**L. Remila:** Resources, Investigation, Validation, Formal analysis, Writing – original draft. **E. Belcastro:** Resources, Investigation, Validation, Formal analysis. **N. Guenday-Tuereli:** Resources, Investigation. **S. Park:** Resources, Investigation, Validation, Formal analysis. **U. Houngue:** Resources, Investigation. **T. Vandamme:** Supervision. **E. Tuereli:** Resources, Investigation. **P. Kerth:** Conceptualization, Supervision. **C. Auger:** Validation, Formal analysis. **V. Schini-Kerth:** Conceptualization, Supervision, Project administration, Writing – review & editing.

## 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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