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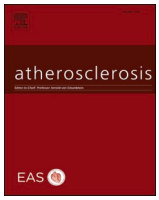

Treatment with APAC, a dual antiplatelet anticoagulant heparin proteoglycan mimetic, limits early collar-induced carotid atherosclerotic plaque development in *Apoe*$^{-/-}$ mice

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ABSTRACT

Background and aims: Mast cell-derived heparin proteoglycans (HEP-PG) can be mimicked by bioconjugates carrying antithrombotic and anti-inflammatory properties. The dual antiplatelet and anticoagulant (APAC) construct administered, either locally or intravenously (i.v.), targets activated endothelium, its adhesion molecules, and subendothelial matrix proteins, all relevant to atherogenesis. We hypothesized that APAC influences cellular interactions in atherosclerotic lesion development and studied APAC treatment during the initiation and progression of experimental atherosclerosis.

Methods: Male western-type diet-fed *Apoe*$^{-/-}$ mice were equipped with perivascular carotid artery collars to induce local atherosclerosis. In this model, mRNA expression of adhesion molecules including ICAM-1, VCAM-1, P-Selectin, and Platelet Factor 4 (PF4) are upregulated upon lesion development. From day 1 (prevention) or from 2.5 weeks after lesion initiation (treatment), mice were administered 0.2 mg/kg APAC i.v. or control vehicle three times weekly for 2.5 weeks. At week 5 after collar placement, mice were sacrificed, and lesion morphology was microscopically assessed.

Results: APAC treatment did not affect body weight or plasma total cholesterol levels during the experiments. In the prevention setting, APAC reduced carotid artery plaque size and volume by over 50 %, aligning with decreased plaque macrophage area and collagen content. During the treatment setting, APAC reduced macrophage accumulation and necrotic core content, and improved markers of plaque stability.

Conclusions: APAC effectively reduced early atherosclerotic lesion development and improved markers of plaque inflammation in advanced atherosclerosis. Thus, APAC may have potential to alleviate the progression of atherosclerosis.

1. Introduction

Atherosclerosis, characterized by the build-up of cholesterol-containing inflammatory plaques in the arterial wall, is the main underlying pathology of acute cardiovascular events, such as myocardial infarction and stroke [1]. The atherothrombotic events occur upon inflamed or erupted endothelium, and ruptured plaques expose deeper thrombogenic layers of the plaques containing collagen and tissue factor [2]. Plaque formation is caused by the influx of lipids and inflammatory cells into the subendothelial space upon repeated endothelial damage, and it is associated with smooth muscle cell proliferation, events which gradually increase upon aging. The thrombogenic hypothesis of Duguid in 1960 was based on the discovery of mural thrombi becoming incorporated in the intima to form layered fibrous thickenings [3].

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Extravasated blood may also carry blood cells, including platelets, plasma proteins, and lipids from vasa vasorum into the atheromatous foci. Later, this hypothesis was complemented by the concept of inflammation being a fundamental element of atherosclerosis [4,5]. Indeed, the all-encompassing concept of thrombo-inflammation currently dominates our understanding of this chronically progressing disease with thrombosis contributing throughout the evolution of the disease process, i.e., from lesion initiation up to formation of a non-occluding or occluding arterial thrombus [5,6].

The mast cell, an immune cell type involved in host defense responses and in allergic diseases and asthma, has also been shown to accumulate in advanced human atherosclerotic plaques [7,8]. In the plaque, mast cells can influence the ongoing disease process by secreting proteases and pro-inflammatory cytokines into their microenvironment [9]. However, mast cells are also the major source of heparin proteoglycans (HEP-PGs) [10]. In the vascular wall, mast cell-derived HEP-PGs can act as local anticoagulants and may counteract the procoagulant effects upon damage. Importantly, *in vitro* experiments have revealed that the HEP-PGs released by stimulated mast cells are capable of inhibiting collagen-induced platelet aggregation uniquely from other agonists. Also, perfusion of blood, spiked with isolated HEP-PG, over a collagen surface inhibits the growth of the platelet-rich thrombus under the high-shear rate and von Willebrand factor (VWF)-mediated conditions [11,12]. HEP-PGs are, thus, antithrombotic both when they are immobilized on collagen surface or when they are present in the blood [11,13]. Importantly, locally applied isolated HEP-PG was shown to inhibit thrombus formation at microsurgical anastomosis sites *in vivo* [14].

Based on the antithrombotic properties of HEP-PGs, we have tailored a semisynthetic bioconjugate with a standardized number of heparin chains onto human serum albumin. This bioconjugate exhibits both antiplatelet and anticoagulant activity (APAC) [15], and it so alleviates ischemic reperfusion injury (IRI) in the kidneys [16], brain [17] and the heart (unpublished data). Due to their rapid targeting to the injured vasculature [18] and small dosing, the APAC conjugates have initially been designed as local antithrombotics for surgical and other intervention strategies to avoid bleeding complications. Importantly, in these settings the primary hemostasis is preserved and several platelet-agonist routes function normally.

Interestingly, APAC was found to be capable of attenuating vascular injury and immune cell activation in a rat model of ischemia-reperfusion induced acute kidney injury [16]. With vascular injury and immune activation being important triggers and promoters of atherogenesis, the above data suggest that APAC may alleviate the progression of atherosclerosis. Other antithrombotic agents have shown a protective profile in animal models of atherosclerosis, which supports this hypothesis. In two independent studies, treatment with rivaroxaban, a factor Xa inhibitor, demonstrated plaque-stabilizing effects in Apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice, by increasing cap thickness [19], and by reducing macrophage and necrotic core content [20]. In clinical use, rivaroxaban, when combined with aspirin to reach inhibition of activity of both coagulation Factor Xa and thromboxane-stimulated platelets, benefitted some patients having severe atherosclerotic peripheral arterial occlusive disease (COMPASS trial) [21].

Our study aimed at assessing the effects of APAC treatment both during initial (prevention setting) and progressing (treatment setting) atherosclerosis in an *Apoe*$^{-/-}$ mouse model with collar-induced lesions, where local hemodynamic alterations play a significant role.

2. Materials and methods

2.1. APAC

APAC, a semisynthetic highly polyanionic bioconjugate of unfractionated heparin (UFH) (7 ± 2 chains, MW appr. 180 kDa) to albumin core was provided by Aplagon Ltd. The APAC complex was characterized by polyacrylamide gel electrophoresis (PAGE; Tris-Glycine 4–20 %, Mini-Protean TGX gel, Bio-Rad, Hercules, CA, USA; 120 V for 15 min and 45 min 180 V for 45 min) under native conditions and compared with unconjugated UFH (Leo Pharma, Ballerup, Denmark), human serum albumin (Biotest AG, Dreieich, Germany), and MW standard (st) (MWP04, Nippon Genetics Europe, Düren, Germany). The gel was stained sequentially with 0.125 % Alcian Blue 8GX (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) in 25 % EtOH, 10 % HAc, and Bio-Safe Coomassie Brilliant Blue G-250 (Bio-Rad) to detect glycosaminoglycan and protein moieties, respectively.

2.2. Animals

All animal experiments were performed in compliance with the guidelines of the Dutch government and the Directive 2010/63/EU of the European Parliament. The experiments were approved by the Ethics Committee for Animal Experiments and the Animal Welfare Body of Leiden University (Project 106002017887), and executed in compliance with the ARRIVE guidelines, taking the 3R principles into account. The outlines of the prevention and treatment setting of the experiments are provided in Supplemental Fig. 1.

Ten to twelve-week-old male *Apoe*$^{-/-}$ mice on a C57BL/6 background (n = 14–15/group) were bred in-house and provided with food and water ad libitum. The mice were fed a cholesterol-rich Western-type diet (0.25 % cholesterol, 15 % cocoa butter, Special Diet Services, Essex, UK), from 2 weeks before (T = −2 weeks) up to 5 weeks after collar placement. At week 0, the mice were randomized into groups based on age, weight, and serum cholesterol levels. Carotid artery plaque formation was induced by bilateral perivascular collar placement in these mice, as described previously [22]. This collar model has previously been shown to provide established atherosclerosis in male hyperlipidemic mice [22]. In this model, mRNA expression of adhesion molecules including ICAM-1, VCAM-1, P-Selectin, and Platelet Factor 4 (PF4) are upregulated upon lesion development, rendering this model highly suitable for our research question.

In short, mice were anesthetized by subcutaneous injection of ketamine (60 mg/kg), fentanyl citrate, and fluanisone (1.26 mg/kg and 2 mg/kg, respectively), after which sedation was monitored by toe pinch. Access to the anterior cervical triangles was gained through a sagittal anterior neck incision and both carotid arteries were carefully dissected free from the surrounding tissue. Silastic collars (Dow Corning) were placed around both carotid arteries and fixed with 3 circumferential silk ties. Subsequently, the entry wound was closed, and the animals were returned to their cage for recovery from anesthesia.

Immediately (initiation) or from week 2.5 weeks (progression) after the collar placement onwards, the mice received either 0.2 mg/kg APAC (Aplagon Ltd, Helsinki, Finland), or vehicle (137 mM NaCl, 10 mM Na_2HPO_4, pH7.5) as control three times per week via intravenous (i.v.) injection into the tail vein for 2.5 weeks (n = 12–14 per group due to some loss of animals after surgery). This dose of APAC was chosen based on earlier observations upon repeated dosing of APAC as described by Craige et al. [23] and own unpublished data. The administered APAC dose (0.2 mg/kg i.v.) was adjusted according to the heparin glycosaminoglycan (GAG) content as defined by a Blyscan Sulfated Glycosaminoglycan Assay (Biocolor Ltd., Carrickfergus, Northern Ireland, UK), and a reference curve for UFH. Blood was collected from the tail vein in weeks 2 and 3. At week 5, the mice were sacrificed upon subcutaneous administration of anesthetics (ketamine (100 mg/kg) and xylazine (10 mg/kg)). Blood was collected via the orbital route, and thereafter the mice were perfused with PBS through the left cardiac ventricle. Upon complete PBS perfusion, organs were collected for analysis. Supplementary Fig. 1 illustrates the administration strategy for this study with the two experimental settings; the prevention and treatment models.

2.3. Cholesterol assay

Serum was collected after centrifugation of blood at 8000 rpm for 10 min at 4 °C and stored at −80 °C until analysis. Total cholesterol levels were determined through an enzymatic colorimetric assay (Roche/Hitachi, Mannheim, Germany). Precipath standardized serum (Roche Diagnostics) was used as an internal standard.

2.4. Cell isolation and analysis

For general white blood cell analysis, blood samples were lysed with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) to obtain a single white blood cell suspension. These were extracellularly stained with a mixture of selected fluorescently labeled antibodies (Supplementary Table 1) for 30 min at 4 °C.

Whole blood samples were collected in citrate-coated tubes and gently mixed with prewarmed PBS (1:1). The whole blood samples were stained with fluorescently labeled antibodies for 15 min at room temperature. Single-cell and whole blood samples were measured with a Cytoflex S (Beckman and Coulter, USA) and analyzed with FlowJo v10.7 (Treestar, San Carlos, CA, USA).

2.5. Histology

After euthanasia, the carotid arteries were dissected, embedded, and frozen in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance CA, USA). Subsequently, 10 μm sections of the carotid arteries were prepared for histological analysis. For each artery, collection of the sections started immediately on the proximal side of the collar, and for each slide, the sections were collected every 90 μm until the complete disappearance of the plaque. This strategy also covers the hemodynamical impact of blood-vessel wall interphase upon the atherosclerotic lesions. Mean plaque size, plaque size at the site of maximal stenosis, plaque volume, and necrotic areas were measured using hematoxylin and eosin (H&E) staining. The collagen content of the plaque was measured using a Sirius Red staining. Macrophage content was determined by using MOMA-2 antibody (1:1000; rat IgG2b; Bio-Rad). Vascular smooth muscle cells were stained using an α-smooth muscle actin antibody (1:1000, IgG2a, Sigma). Mast cells and neutrophils were manually counted after staining slides using a Naphthol AS-D Chloroacetate Specific Esterase Kit (Sigma). All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

2.6. RNA isolation, cDNA synthesis, and qPCR

Three to four carotid artery plaque segments, obtained at 5 weeks after collar placement, were pooled and homogenized in guanidine thiocyanate (GTC) using as tissue homogenizer, after which total RNA was extracted (n = 4 samples per group). RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas). Quantitative analysis of genes (Supplementary Table 2) was performed with a 7500 fast real-time PCR system (Applied Biosystems).

2.7. Plasma coagulation times

Prior to the atherosclerosis studies, *Apoe*$^{-/-}$ mice (n = 3/group) administered with 0.2 mg/kg APAC, 0.2 mg/kg unfractionated heparin (UFH), or vehicle and blood was collected by retro-orbital bleeding at 15 min after dosing. Activated partial thromboplastin time (APTT) (SynthaSIL, IL Werfen, Germany) and thrombin time (TT) (STA Thrombin2, Stago, France) were assessed from citrated (3.2 %) plasma obtained after centrifugation (8000 rpm).

2.8. Statistical analysis

Data were analyzed using Prism 9.0 (GraphPad Software, Inc. San Diego, CA, USA), and are expressed as mean ± SEM for all analyses. The Shapiro-Wilkson normality test was used to test data for normal distribution. Normally distributed data were compared with an unpaired two-tailed Student t-test. When data were not normally distributed, a Mann-Whitney *U* test was used. Probability values of $p < 0.05$ were considered significant.

3. Results

The APAC conjugation reaction was characterized by PAGE analysis under non-reducing conditions. Non-conjugated UFH and HSA showed distinct migration patterns in comparison with APAC and in relation to molecular weight standards (Fig. 1). APAC effects on plasma coagulation times were assessed separately from the main study in *Apoe*$^{-/-}$ mice administered with 0.2 mg/kg of APAC, UFH, or vehicle. Neither APAC nor UFH, after a single intravenous administration at this concentration, affected plasma coagulation times in the *Apoe*$^{-/-}$ mice (Supplementary Fig. 2).

3.1. APAC prevents early development of atherosclerosis

In the prevention setup, we assessed the effects of APAC administration (0.2 mg/kg i.v.) on the initial lesion induction and development. APAC was administered immediately after placement of the collar to induce atherosclerosis in *Apoe*$^{-/-}$ mice (Supplementary Fig. 1). APAC did not affect total body weight or plasma total cholesterol levels during

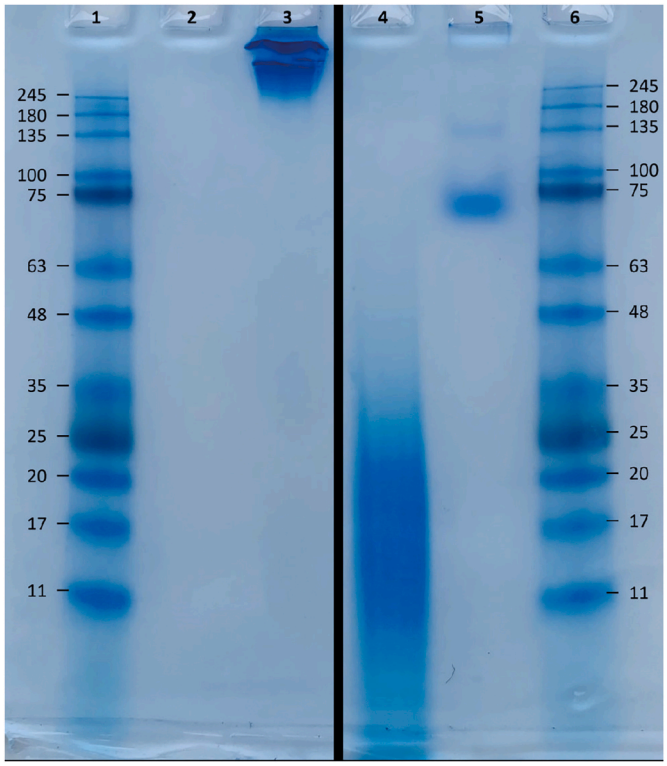

Fig. 1. APAC is a bioconjugate between HSA and UFH.
Conjugation of UFH chains to HSA in APAC is depicted by PAGE analysis under native, non-reducing, conditions: 1. and 6. MW st, 2. PBS buffer control, 3. APAC (4 μg; the heparin equivalent quantity), 4. UFH (5 μg), and 5. HSA (2 μg). Tris-Glycine 4–20 % gel, stained with Alcian Blue and Coomassie Brilliant Blue to detect glycosaminoglycan and protein moieties, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the study (Supplementary Figs. 3A and B). Also, APAC treatment during the first 2.5 weeks of lesion development did not influence the percentage of circulating myeloid populations, including monocytes and neutrophils (Supplementary Figs. 3C and D), or the B and T lymphocytes (Supplementary Figs. 3E, F, G) at the endpoint of the study.

Administration of APAC during lesion initiation reduced plaque development ($p < 0.01$) at the sites of maximal stenosis: the size of the plaques was more than 50 % smaller compared to the control group (Fig. 2A and B). This observation was also translated into a smaller average lesion size throughout the collar-induced plaque (Fig. 2C, D, $p < 0.01$). Consequently, the total plaque volume was diminished by 60 % ($p < 0.05$, Fig. 2E) in the APAC-treated mice. The medial layer was not impacted by APAC at the site of maximal stenosis (Fig. 2F), but the total vessel area was significantly inhibited by APAC treatment (control: 1.6 $\pm$ 0.1*10^5 μm^2 *versus* APAC: 1.2 $\pm$ 0.1*10^5 μm^2, $p < 0.05$, Fig. 2G), suggesting that APAC treatment prevented outward remodeling to a certain extent.

Further analysis of plaque morphology revealed that the average macrophage areas in the plaques tended to reduce upon APAC treatment ($p = 0.05$, Fig. 3A and B), while the macrophage content relative to lesion size remained unchanged (Fig. 3C). Similarly, both absolute collagen ($p < 0.01$, Fig. 3D–F) and necrotic areas ($p < 0.05$, Fig. 3G and H) were decreased in the APAC-treated mice *versus* controls significantly by over 50 %. Vascular smooth muscle cell content, both absolute and relative to lesion size, did not differ between the groups (Supplementary Figs. 4A and B). As the values relative to lesion size were not altered, our data suggest that lesions in the APAC treated group represent an earlier stage of development than in the control plaques, and that APAC thus inhibited overall lesion development. APAC treatment did not influence the mast cell numbers or its activation status in the perivascular tissue (Fig. 3I). In addition, the numbers of neutrophils in the intima seemed somewhat, but not significantly, reduced (Fig. 3J).

3.2. APAC reduces markers of inflammation in advanced plaques

In the treatment set-up, we aimed to assess the effects of APAC treatment on the progression of the already developing atherosclerotic lesions at 2.5 weeks after the initiation of collar-accelerated carotid artery atherosclerosis (Supplementary Fig. 1). As in the previous experiments, APAC did not influence total body weight or plasma total cholesterol levels (Supplementary Figs. 5A and B). Circulating white blood cell and total platelet counts remained unaffected as well (Supplementary Figs. 5C-H).

Treatment with APAC upon lesion progression did not significantly affect the carotid plaque size at the site of maximal stenosis (Fig. 4A and B). Also, the average lesion size throughout the carotid artery plaque did not significantly differ between the groups (Fig. 4C). APAC-treated lesions tended to progress less along with increasing distance from the collar placement (Fig. 4D). Indeed, plaque volume tended to be decreased, although not significantly, in the APAC-treated group ($p = 0.1$, Fig. 4E), and so did the average plaque length when expressed in the distance proximal from the collar ($p = 0.07$, Fig. 4F). In the treatment setting, APAC treatment did not impact either the surface area of the medial layer (Fig. 4G) or total vessel area (control: 1.4 $\pm$ 0.2*10^5 μm^2 *versus* APAC: 1.3 $\pm$ 0.1*10^5 μm^2, Fig. 4H) at the site of maximal stenosis.

However, interestingly, macrophage area was significantly reduced with 42 % in the APAC-treated group ($p < 0.05$, Fig. 5A–C). While the collagen content was unaffected (Fig. 5D–F), the absolute necrotic core size tended to reduce ($p = 0.07$, Fig. 5G). As in the prevention setting, APAC treatment did not affect smooth muscle cell content (Supplementary Figs. 6A and B). Relative to the lesion size, the necrotic area diminished by 25 % ($p < 0.05$, Fig. 5H). An increased collagen/necrotic area-ratio (APAC: 0.85 $\pm$ 0.06 *versus* 0.57 $\pm$ 0.07, $p < 0.01$, Fig. 5I) suggests that, overall, APAC treatment improved markers of plaque stability upon lesion progression. The reduction in macrophage content coincided in particular with reduced relative Tnfα mRNA expression levels in the carotid artery plaque (relative expression; control: 8 $\pm$ 2*10^{-3} *versus* APAC: 4 $\pm$ 2*10^{-3}, $p < 0.05$, Fig. 5J), illustrative of reduced inflammatory signaling via TNFα. The gene expression of other inflammatory cytokines such as Ccl2, Il-1β and Il-6 were not affected by APAC treatment (Supplementary Table 3). In addition, carotid plaque gene expression analysis of phenotypic macrophage

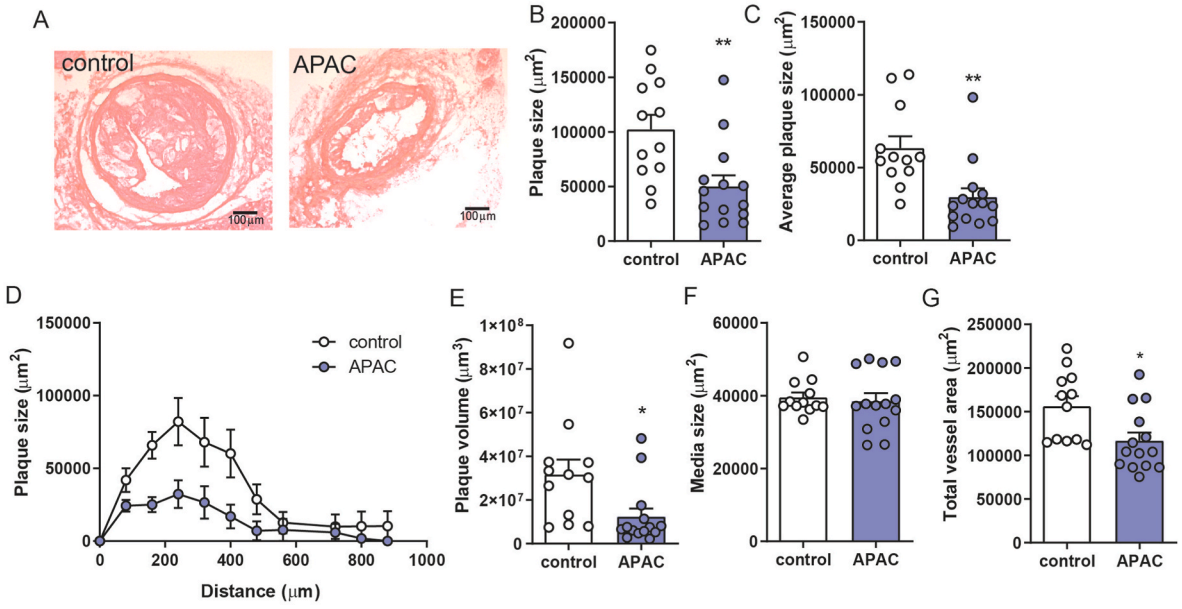

Fig. 2. In the prevention setting APAC inhibits atherosclerotic lesion initiation.
(A) Representative H&E staining images of collar-induced atherosclerotic plaques observed in control and APAC-treated *Apoe*$^{-/-}$ mice. (B) APAC reduced collar-induced atherosclerosis at the site of maximal stenosis. (C) APAC reduced the average sizes of the lesions over 50 %. (D) Distance analysis revealed smaller atherosclerotic lesions in APAC-treated mice than in controls throughout the carotid artery plaque, resulting in (E) a strong reduction in plaque volume. (F) Media size did not differ between the APAC and control groups. (G) Total vessel area at the site of maximal stenosis was reduced upon APAC treatment. N = 12–14, data are given as mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$.

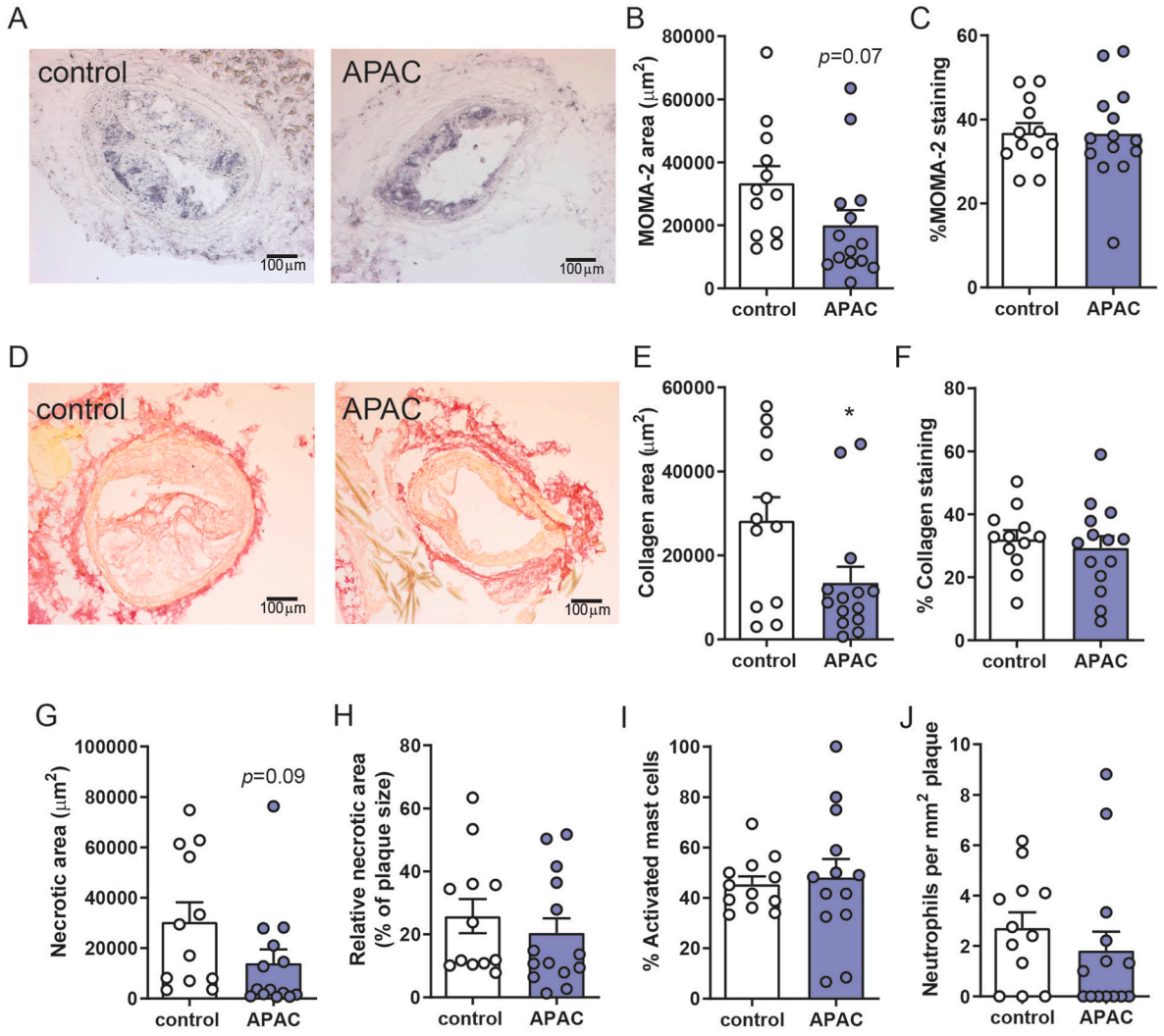

Fig. 3. Plaque morphology analysis in the APAC prevention setting.
(A) Representative images of MOMA-2 staining, showing macrophage positive areas in the carotid artery plaques. (B) Absolute macrophage area tended to be reduced upon APAC treatment compared to controls. (C) Relative to plaque size, macrophage content did not differ between the groups. (D) Collagen content in the carotid artery plaques was measured using a Sirius Red staining, of which representative images of the control and APAC groups are displayed. (E) Absolute collagen area was significantly smaller in plaques of the APAC-treated mice compared to controls, (F) while the collagen content relative to lesion size was similar between the groups. APAC tended to reduce the necrotic core area of the plaque both in the absolute (G) and relative (H) values. (I) Percentage of activated mast cells in the perivascular tissue. (J) Neutrophil numbers in the plaque. N = 12–14, data are given as mean ± SEM. *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

markers such as Ccr2, Mrc1 or Arg1 did not reveal differences, suggesting that APAC did not influence the macrophage phenotype (Supplementary Table 3). Along the prevention setting, neither the activation status of perivascular mast cells (Fig. 5K) nor the neutrophil numbers (Fig. 5L) differed between the groups.

4. Discussion

In the present study, we aimed to assess the efficacy of APAC, a potent antiplatelet and anticoagulant treatment modality, in the inhibition of atherosclerotic lesion development and progression in a mouse model, in which the endothelium is activated due to the presence of hyperlipidemia and increased oscillatory shear stress [22]. First, we confirmed that APAC indeed is a heparin-proteoglycan mimetic, based on the bioconjugation between the UFH chains to HSA core. *In vivo*, we showed that APAC reduced initial atherosclerotic lesion formation and limited inflammation in pre-existing lesions in a therapeutic setting, independently of plasma cholesterol levels, as summarized in Fig. 6. As local thrombotic responses and inflammation are seminal pathogenic features in tissue injury and atherogenesis [1,4,5], the present findings support the vascular reprogramming potential of APAC in these pathological processes.

In models of arterial injury triggered by balloon angioplasty and arteriovenous fistula surgery, we have previously shown that local APAC administration colocalizes with VWF, collagen, and laminin in areas with endothelial loss [18]. Also, in mice subjected to a laser-induced carotid injury, the arterial occlusion time was prolonged by APAC in comparison with UFH, and in a collagen-exposing carotid surgery model, APAC rapidly binds to the extracellular matrix, and upon such binding the platelet thrombus formation is practically abolished [24]. In an IRI model of kidney injury in rats [16] and in myocardial injury in mice (unpublished), a single intravenously administered dose of APAC (0.13 or 0.5 mg/kg, respectively) has shown strong tissue-protective and anti-inflammatory properties. Therefore, the unique and multifunctional properties of APAC, including the targeting and binding to arterial injury sites and the preservation of its functionality in situ, render this

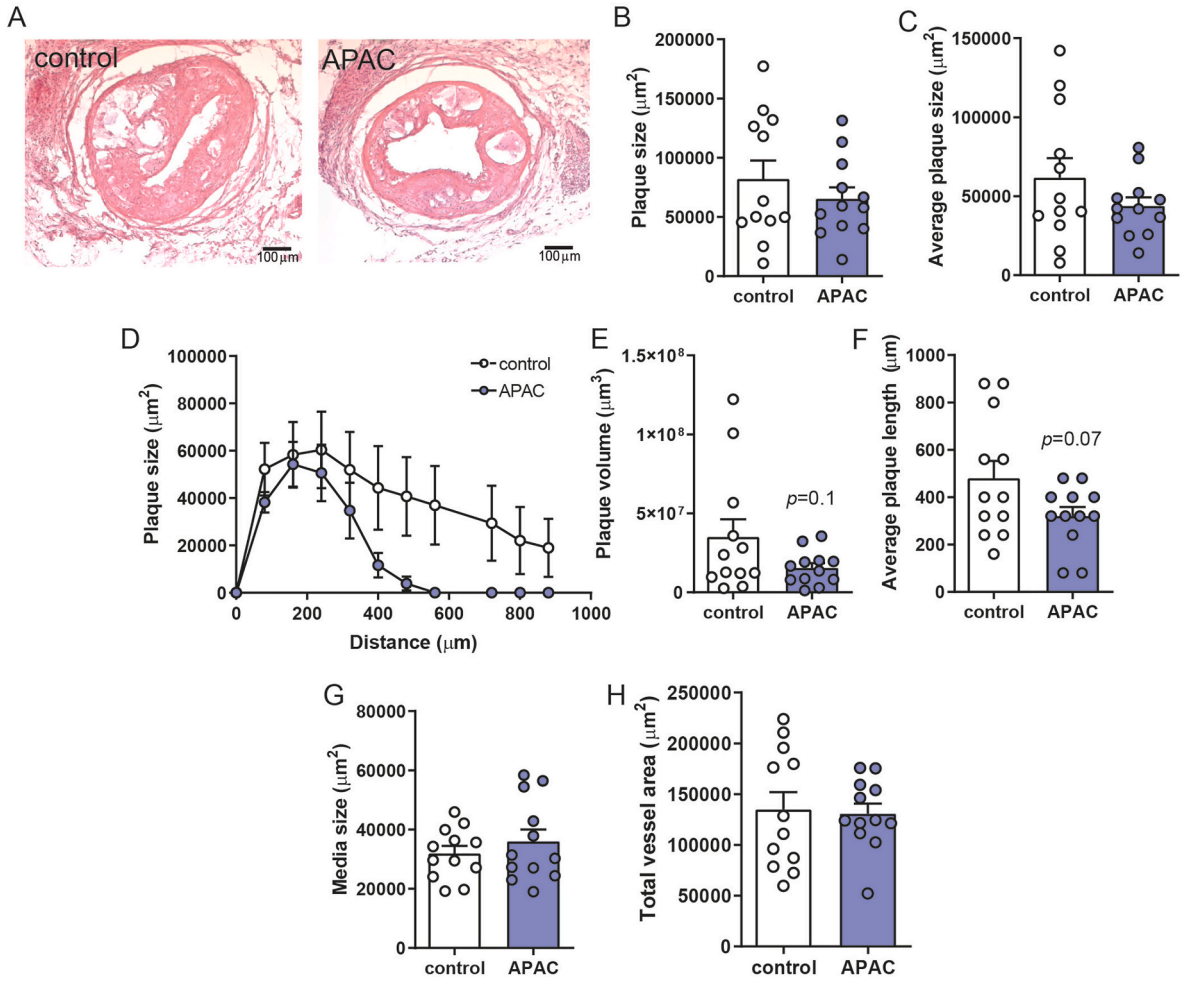

Fig. 4. In the treatment setting APAC attenuated atherosclerosis progression.
(A) Representative H&E staining images of collar-induced atherosclerotic plaques observed in control and APAC-treated *Apoe*$^{-/-}$ mice during lesion progression. (B) APAC treatment during lesion progression did not affect collar-induced atherosclerosis at the site of maximal stenosis or on (C) average lesion size level. (D) Distance analysis revealed a tendency to smaller atherosclerotic surface area in the APAC group compared to the controls when the distance from the collar increased. (E) Plaque volume was not significantly reduced, however, in (F), the average plaque length tended to be reduced upon APAC treatment. (G) Media thickness and (H) Total vessel area did not differ between the APAC and the control group. N = 12–14, data are given individually and as mean ± SEM. N = 12–14 per group, data are given as mean ± SEM.

compound capable of locally attenuating platelet-collagen interactions, and thrombin and fibrin formation at the injured tissue locations [15,18,24,25]. In the current study, we now also firmly establish that APAC protects against early atherosclerosis development which is reflected by the presence of plaques with a less advanced phenotype. In more advanced lesions, plaque inflammation was limited by APAC, as illustrated mainly by a reduced macrophage content as well as by the reduced size of the necrotic core.

We did not observe prolongation of APTT or thrombin time after a single intravenous injection with either APAC or UFH in *Apoe*$^{-/-}$ plasma, unlike previously in normal mouse plasma [23]. Although APTT and TT after repeated APAC administration remain to be assessed in our mouse model, hyperlipidemia in humans is associated with enhanced thrombin generation [26], which may explain the lack of effect in our hyperlipidemic mice. The underlying mechanisms via which APAC protects against lesion initiation and stabilizes more established lesions remain to be better understood, although previous studies using anticoagulants have provided evidence for anti-inflammatory pathways also to be involved. For example, as has been described for the FXa inhibitor rivaroxaban [19,20], we observed reduced plaque inflammation in the prevention but most prominently in the treatment study. Also, double knock-out models of *Apoe*$^{-/-}$ and FVIII or FXI show that coagulation factors reduce the development of atherosclerosis, due to for example a reduction in plaque macrophage content [27,28]. We also demonstrated a reduced macrophage content, suggesting that APAC may inhibit the influx of pro-inflammatory monocytes into the lesion. This may occur via a reduction of the inflammatory mediators TNFα, IL-6 and MCP-1. Indeed, we observed a reduction particularly in TNFα mRNA expression in the plaques of the therapeutic study, supporting this notion at least partially. The exact underlying mechanisms that lead to the reduced inflammation remain to be investigated. Based in our gene expression analysis of the atherosclerotic plaques, there is no indication for an alteration in the macrophage phenotype by APAC treatment. We hypothesize that APAC may act anti-inflammatory via downregulation or shielding of adhesion molecules such as P-selectin and PF4. Previously, APAC has been shown, via its polyanionic charge, to have a strong binding affinity to multiple positively charged blood and extracellular matrix proteins, which are involved in progression of atherosclerosis, including VWF, neutrophil binding chemokine PF4 [29] and P-selectin [30], as has been reported also in the cancer setting. Overall, the limited influx of inflammatory cells into the vascular wall did result in a reduction in the necrotic core formation, which we observed as the most prominent APAC-dependent effect in the progression study.

We should consider the possibility that APAC, which can bind LDL-

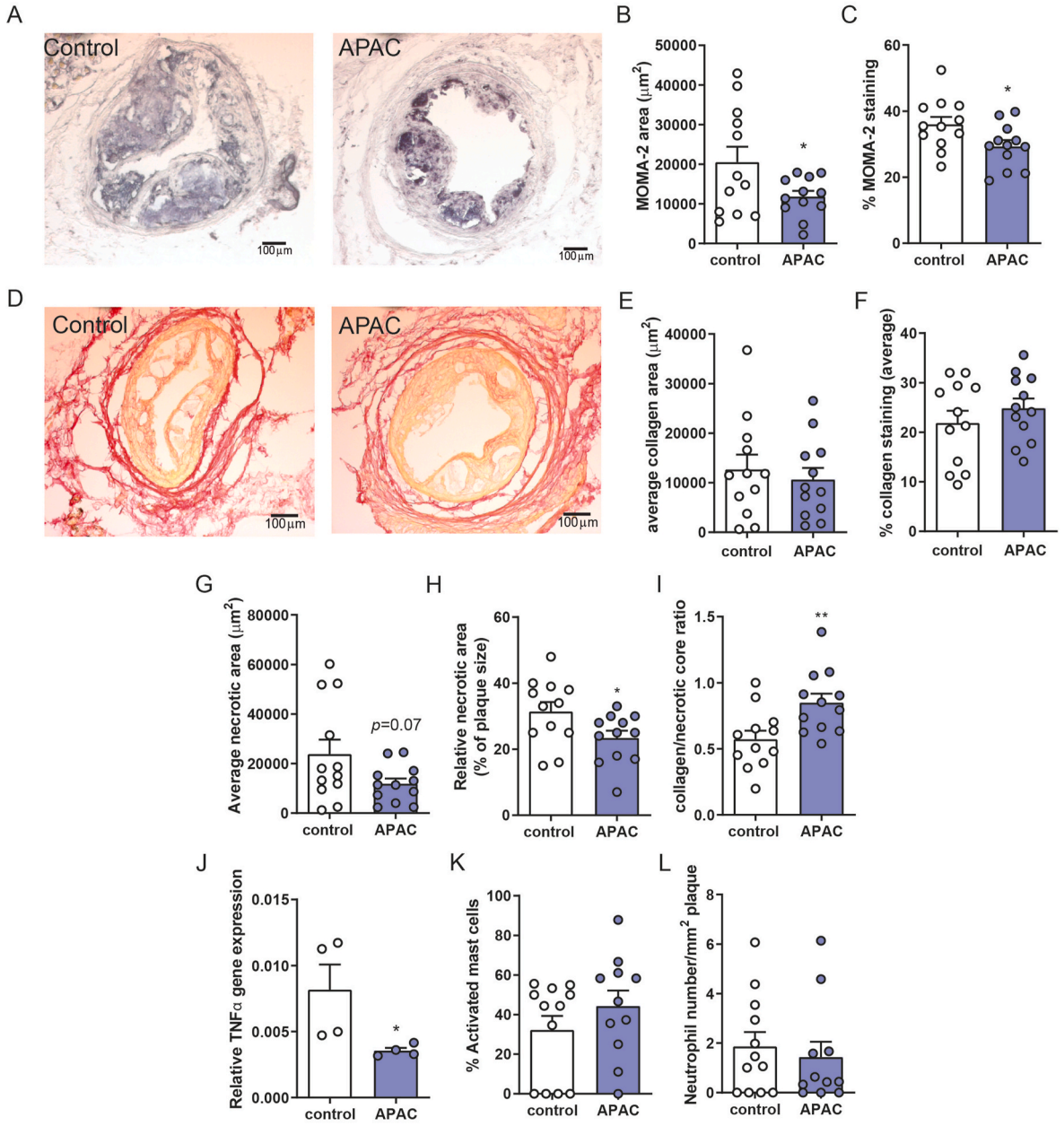

Fig. 5. Plaque morphology analysis in the APAC treatment setting.
(A) Representative images of MOMA-2 staining show macrophage-positive areas in the carotid collar-induced atherosclerosis in both groups. Macrophage content was significantly reduced by APAC treatment compared to controls, both in the absolute area (B) and (C) relative to the plaque size. (D) Collagen content of the carotid artery plaques was analyzed using a Sirius Red staining, and representative images of the control and APAC groups are shown. (E) Absolute collagen area did not differ between plaques of the APAC-treated mice and the controls, and the content relative to lesion size was also similar (F). The necrotic core area of the plaque was reduced in the absolute values (G), which were significant relative to lesion size (H). (I) The collagen/necrotic core ratio was significantly increased in the APAC group. (J) Relative TNFα gene levels in pooled carotid artery plaques. (K) Percentage of activated mast cells in the perivascular tissue and (L) Neutrophil numbers in the plaque were similar. N = 12–14, data are given as mean ± SEM. *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

particles via ionic interaction with the positively amino-acid residues of the apoB-component of the particles, may compete with arterial proteoglycans for LDL-binding in the arterial wall. This would reduce the retention and modification of the LDL-particles in the plaque, leading to reduced foam cell formation and necrotic core formation. This is relevant when considering the pathogenesis of atherosclerosis, in which lesion formation is characterized by both extra- and intracellular accumulation of lipids. These lipids, derived from apolipoprotein B (apoB)-containing lipoproteins that have entered the arterial intima, bind to the proteoglycan structures in the arterial wall and then become modified by oxidative agents and hydrolytic enzymes [31]. Such modified lipoprotein particles are subsequently recognized and ingested by macrophages that differentiate into foam cells, which ultimately die and participate in the formation of the necrotic lipid core [32]. Via lipoprotein retention, APAC may thus limit this process, provided it reaches the sites of endothelial activation or the subendothelial space upon increased shear stress, prevalent in our collar model.

Although we do not observe plaque rupture in our mouse model of atherosclerosis, we do not exclude local effects of APAC on atherothrombosis. This is because our data closely relate to studies

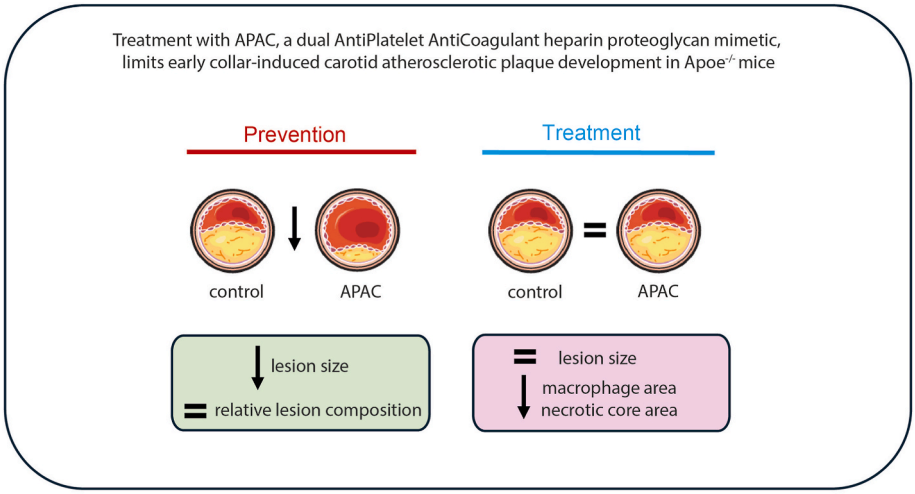

Fig. 6. Graphical abstract with a summary of the main study findings.

investigating the role of VWF in atherogenesis. In the 1980s, Fuster et al. observed that homozygous VWF-deficient pigs were almost devoid of aortic atherosclerosis, while control pigs with normal VWF levels expressed marked atherosclerosis [33]. In another study, the involvement of VWF in atherogenesis was examined in hypercholesterolemic LDL receptor-deficient mice by breeding them with VWF-deficient mice [34]. Half of the aortic lesions were located at the branch points of the renal and mesenteric arteries where disturbed blood flow exists. Importantly, the lesions at these sites were 40 % less prominent in the VWF-negative mice, quite similar to our collar-injured carotid lesions in the Apoe$^{-/-}$ mice. The authors suggested that VWF deficiency may have reduced platelet and leukocyte adhesion to the lesion sites and/or attenuating mechano-transduction pathways of shear forces to the endothelium in the vascular wall. These prior results coincide well with those of our earlier work, particularly since, by using human blood, we could previously demonstrate significant inhibition of high shear-induced VWF-mediated thrombus growth both with APAC and its parent molecule of heparin proteoglycans [11,25]. Closing the loop back to atherothrombosis and inflammation, the many VWF-mediated mechanisms of platelet-vessel wall interactions which involve adhesion molecules, represent therapeutic targets in the early phases of thrombo-inflammatory atherogenesis [35–39].

Altogether, as summarized in the graphical abstract, the protective features of APAC against atherothrombosis, and particularly inflammation, are likely to underlie the efficacy of APAC in experimental atherosclerosis, not only in preventive but also in treatment settings.

CRediT author contribution statement

Ilze Bot: Conceptualization, executing experiments, data acquisition, Formal analysis, and interpretation, Writing – original draft, Funding acquisition. Lucie Delfos: data acquisition, Formal analysis, writing – review, and editing. Esmeralda Hemme: data acquisition, writing – review, and editing. Mireia Bernabé Kleijn: executing experiments, data acquisition. Peter van Santbrink: executing experiments, data acquisition. Amanda Foks: data acquisition, writing – review, and editing. Petri Kovanen: Conceptualization, Supervision, writing – review, and editing. Annukka Jouppila: Development of APAC, Conceptualization, Formal analysis and interpretation, Writing – original draft. Riitta Lassila: Invention and development of APAC, Conceptualization, Supervision, writing – review, and editing.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

RL is the CSO and CMO of Aplagon Ltd. The other authors declare no competing interests.

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Appendix A. Supplementary data

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