Eur J Vasc Endovasc Surg (xxxx) xxx, xxx

Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model

Karina A. Barreiro [a,f,1], **Riikka Tulamo** [b,1], **Annukka Jouppila** [c], **Anders Albäck** [b], **Riitta Lassila** [d,e,f,*]

[a] Institute for Molecular Medicine Finland FIMM, University of Helsinki, Helsinki, Finland
[b] Department of Vascular Surgery, Helsinki University Hospital, University of Helsinki, Helsinki, Finland
[c] Helsinki University Hospital Clinical Research Institute, Helsinki, Finland
[d] Coagulation Disorders Unit, University of Helsinki, Departments of Haematology and Clinical Chemistry (HUSLAB Laboratory Services), Comprehensive Cancer Center, Helsinki University Hospital and Research Program in Systems Oncology, Faculty of Medicine, University of Helsinki, Helsinki, Finland
[e] Helsinki University, Faculty of Medicine, Research Program in Oncology, Helsinki, Finland
[f] Aplagon Oy, Helsinki, Finland

WHAT THIS PAPER ADDS

This experimental study on pigs shows that both *ex vivo* and *in vivo*, locally administered dual antiplatelet and anticoagulant (APAC) targets the vascular injury sites under normal and high flow conditions in an acute setting. In the vein and in the balloon injured artery, APAC also penetrates the vessel wall. The binding counterparts include internal elastic lamina, von Willebrand factor, and laminin. These characteristics render APAC a promising novel substance/therapy for both endovascular and open vascular surgery to resist excessive local platelet aggregation and acute clotting at the site of endothelial injury.

Objectives: Vascular binding of dual antiplatelet and anticoagulant (APAC) was assessed in surgically created femoral arteriovenous fistula (AVF) and iliac and carotid artery injury in porcine models.

Methods: Three models of collagen exposing injury were used: 1) femoral AVF, 2) *in vivo* iliac and carotid artery balloon angioplasty injury, and 3) *in vitro* femoral artery endothelial denudation injury. Biotinylated APAC (0.5 mg/mL) was incubated with the injury site before releasing blood flow. APAC, von Willebrand factor (vWF), laminin, platelet endothelial cell adhesion molecule 1 (PECAM-1), and podocalyxin were detected in histological sections using immunofluorescence and confocal microscopy and Manders' co-localisation coefficient (M1).

Results: APAC bound to AVF at anastomosis and to both *in vivo* and *in vitro* injured arteries. APAC co-localised with matrix vWF (M1 $\geq$ 0.66) and laminin (M1 $\geq$ 0.60), but less so if endothelial PECAM-1 or podocalyxin was present (M1 $\leq$ 0.25). APAC targeted and penetrated the injured vessel wall, especially the AVF vein.

Conclusions: APAC, compatible with its high negative charge, rapidly targets injured vessels co-localizing with matrix vWF and laminin, but not with endothelial PECAM-1 and podocalyxin. This localising feature may have potential antithrombotic implications for vascular interventions.

Keywords: APAC, Arteriovenous fistula, Balloon angioplasty, Porcine vascular injury model, Von Willebrand factor
Article history: Received 23 May 2018, Accepted 23 May 2019, Available online XXX

INTRODUCTION

Endothelial injury leads to adhesion and site specific activation of platelets and the coagulation cascade, resulting in local thrombus formation.[1–3]

In vascular procedures, local iatrogenic vascular injury at the site of an intervention is unavoidable and interaction with blood and its constituents will follow to initiate healing. The extension of blood coagulation is prohibited by systemic anticoagulation with a weight adjusted intravenous infusion of unfractionated heparin (UFH) and platelet inhibitors.[4]

Often, vascular patients receive pre-operative oral antiplatelet therapy (i.e. aspirin or clopidogrel) as secondary prevention.[5,6] Currently, as locally administrable antiplatelet regimens are not available for clinical use, systemic exposure to antiplatelet agents together with heparins or other anticoagulants increases peri- and post-operative challenges to haemostasis.[7–9]

[1] Karina A. Barreiro and Riikka Tulamo contributed equally to this paper.

* Corresponding author. Coagulation Disorders Unit, University of Helsinki, Departments of Haematology and Clinical Chemistry (HUSLAB Laboratory Services), Comprehensive Cancer Centre, Helsinki University Hospital, PO Box 372, 00290 Helsinki, Finland.
E-mail address: riitta.lassila@hus.fi (Riitta Lassila).

1078-5884/
https://doi.org/10.1016/j.ejvs.2019.05.019

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

Semisynthetic antiplatelet and anticoagulant (APAC), with its dual action, is a heparin proteoglycan (HEP-PG) mimic designed for vascular interventions.[7,8,10] APAC comprises UFH chains conjugated to a protein core, rendering APAC a unique, locally acting antithrombotic. APAC specifically inhibits collagen induced platelet aggregation and subsequent thrombus growth, but maintains haemostasis, the key initiator of healing after vascular injury. APAC inhibits mainly the intrinsic pathway of coagulation and induction of thrombin generation on platelet surfaces, reducing fibrin formation at the site of arterial injury, thus acting as an anticoagulant.[10] The pharmacokinetics of systemically administrated APAC have been defined in rats,[11] and the benefits of locally administered APAC are described in two short term (one to two hours) baboon arterial injury models and in long term (two weeks) healing of rat arterial anastomosis.[10] Interestingly, intravenously administered APAC also shows significant vasculoprotective effects by preventing ischaemic kidney injury.[10,11]

The injury associated antithrombotic effects allows this therapeutic agent to control collagen and thrombin mediated platelet aggregation and thrombus formation at the site of a vascular intervention. Although APAC has shown its antithrombotic potential in earlier studies, the vascular interaction and binding sites are unknown. Several models of small and large animals have been developed for vascular injury research, but porcine vascular injury carries histological changes best reflecting those described in humans.[12,13] Thus, in the present study, the porcine model was chosen to investigate early binding and co-localisation of locally administered APAC at the site of an experimental human like balloon angioplasty and arteriovenous fistula (AVF) before entering human studies.[14]

METHODS

For a detailed description of materials and methods, see the Supplementary Material. In brief, *in vivo* two injury models were used: 1. the creation of an AVF between the femoral artery (FA) and vein (FV) to mimic open vascular surgery under high blood flow conditions, and 2. balloon injury of iliac (IA) and carotid arteries (CA) to mimic endovascular surgery/procedures. *In vitro* in the absence of blood, the arterial injury site was incubated with APAC to assess direct vascular binding. All animal procedures were performed on female landrace pigs ($n = 5$, mean weight 30 kg) under general anaesthesia with the approval (ESAVI-7811-4.10.2016) of the Regional State Administrative Agency for Southern Finland and in accordance with the guidance and legislation for animal experimentation. An overview of the experiments performed is presented in Table S1.

Blood cell counts and activated partial thromboplastin time (APTT) were analysed for all animals. Animals received peri-operative UFH and one animal was pre-dosed with 100 mg of aspirin to test the haemostatic effect of its co-administration in the model. An AVF was created bilaterally end to side between the FA and FV, and the anastomotic area was incubated with either phosphate buffered saline (PBS) or APAC-biotin conjugate (0.25—0.3 mg in 0.5—0.6 mL of PBS) (henceforth APAC) (Aplagon Ltd., Helsinki, Finland, www.aplagon.com). Thirty minutes after restoring the circulation, the anastomosis area was resected, flushed with saline, and fixed in 10% formalin for histology. Also, a segment of the FA was collected, mechanically de-endothelialised, and incubated in PBS or APAC *in vitro* for two minutes, post-fixed in 10% formalin for six hours, snap frozen in liquid nitrogen, and stored at −80 °C.

For endothelial denudation, a percutaneous transluminal angioplasty (PTA) balloon (Abbott Armada 35, 6 × 40 mm; 50% oversized) was advanced to the mid portion of the common IA or CA, inflated to 6 Atm and moved and rotated 10 times back and forth. Next, 3 mL of PBS (always first) or of APAC (1.5 mg) was slowly released to the denuded area within two minutes, through a maximally filled OTW ClearWay diffusion balloon catheter (6 × 40 mm for IA and 2 × 20 mm for CA; Atrium Europe B.V., SL Mijdrecht, The Netherlands). After 30 min of circulation, the arterial segment was resected and post-fixed as AVF (Fig. S1).

For histology, 10 µm cryo-sections and haematoxylin eosin staining were used. For immunofluorescent staining, APAC was detected with streptavidin-eFluor 660 (50-4317, eBioscience Inc., Affymetrix Inc., San Diego, CA, USA) or streptavidin-ATTO647 (Immune Biosolutions, Sherbrooke, QC, Canada), and antibodies for von Willebrand factor (vWF), laminin, platelet endothelial cell adhesion molecule 1 (PECAM-1), or podocalyxin were used and detected with corresponding secondary antibodies.

Samples were analysed with confocal microscopes of Leica (TCS CARS SP8) or Zeiss (LSM 780). Co-localisation for directly bound APAC and vWF, laminin, or PECAM-1, was analysed with the plug in coloc2 for imageJ (NIH, USA) and Mander's method to estimate co-localisation.[15] Maximum projection images and enhanced contrast (linear adjustment, ImageJ) were used for figure panels.

Statistics

Blood cell counts are expressed as change (%) relative to the baseline value, and APTT as mean(s) ± standard deviation (SD). The reported Manders' co-localisation coefficients M1 (M1) were background corrected and expressed as mean ± standard error of the mean (SEM). A one tailed paired samples *t* test was conducted to compare M1 for APAC-vWF with APAC-PECAM-1, in both AVF and balloon injuries, and differences were analysed with IBM SPSS statistics V24. A p value of ≤ 0.05 was considered statistically significant.

RESULTS

Blood cell counts and coagulation

At baseline, the range of blood cell counts was normal, but they decreased at the end of the four to five hour experiments, mostly platelet counts, by 35% (Table S2), reflecting their consumption during the study.

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

APTT was used to assess the systemic effects of IV UFH bolus, excluding those of local APAC administration. Baseline APTT was 15.6 ± 3.2 s (mean ± SD; $n = 5$), and 10 min after UFH, APTT was prolonged on average by 1.7 ± 0.6-fold (26.4 ± 10.3 s; one to three boluses per animal; $n = 12$) *vs.* the baseline. APTT reverted to baseline values 90 min after each UFH bolus administration. Excluding any systemic effect, the local APAC did not influence APTT (up to 0.3 mg in AVF, APTT 14.9 ± 2.9 s; $n = 5$; or 1.5 mg in balloon injury, APTT 14.7 ± 0.9 s; $n = 3$). The co-administration of aspirin with APAC did not influence APTT or haemostasis (data not shown).

APAC targets and binds to AVF walls

Under this very high flow (290–590 mL/min) AVF model, APAC covered broad areas, including the artery, the vein, and the suture lines (Fig. 1). In the artery, APAC tightly followed the luminal side, reaching the internal elastic lamina. In the vein, APAC covered the luminal side, but also clearly penetrated the deeper layers of the tunica media.

APAC targets and binds to balloon injured artery walls

In the *in vivo* balloon injury models, APAC decorated the luminal side in close contact with the internal elastic lamina, compatible with the binding *in vitro* (Supplementary Figs. S2A–F, and S3A-C). In the control (PBS) artery, the signal was negative (Figs. S2G–I). In CAs, the lesion produced by balloon injury seemed larger than in IAs, and the APAC signal was traced deeper in the tunica media (Figs. S2D–F).

Direct binding of APAC to in vitro *denuded artery*

In the *in vitro* model of denuded FA, illustrating the direct interaction of APAC without contact with blood flow, the APAC signal was detected on the luminal side, next to and following the contour of the internal elastic lamina (Fig. S3A–C). In the control (PBS) artery, the signal was absent (Fig. S3D–F). vWF was absent or barely detectable in *in vitro* denuded arteries (data not shown).

APAC co-localised with vWF in both AVF and balloon injury models

To assess APAC's *in vivo* molecular interactions, the focus was on vWF. On the arterial side of the AVF, 66% of the APAC signal co-localised with vWF (M1 = 0.66 ± 0.03; $n = 3$) (Fig. 2). The absence of any luminal nuclei in the internal elastic lamina (Fig. 2A–D), reflected a lack of endothelium at the binding sites. In the *in vivo* balloon angioplasty, the APAC signal again co-localised with vWF in 70% of the acquisitions (M1 = 0.70 ± 0.05; $n = 3$) (Fig. 2) in CAs, confirming the strong interaction. On the venous side, a similar co-occurrence was noted (Fig. S4), in addition to the deeper penetration of APAC in the vascular wall.

APAC co-localised with laminin in both AVF and balloon injury models

To study the targeting of APAC in subendothelial structures, the co-localisation between APAC and laminin, the major component of the basement membrane, was assessed. On the arterial side of the AVF and in the balloon angioplasty of CAs, about

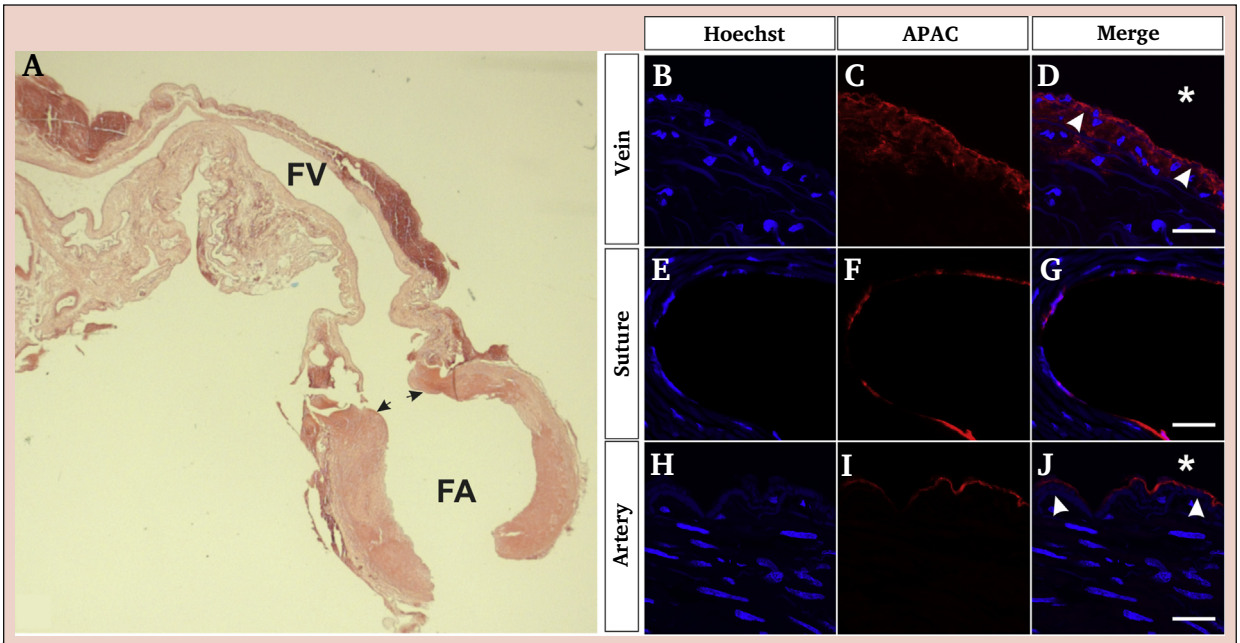

Figure 1. Dual antiplatelet and anticoagulant (APAC) distribution in femoral arteriovenous fistula (AVF) wall in pig. AVF was incubated with APAC before opening the circulation. Haematoxylin eosin staining at the end-to-side anastomosis site (arrows) between the femoral artery (FA) and femoral vein (FV, A) and the corresponding fluorescent stains of luminal parts of vascular walls of vein (B-D), suture site (E-G) and artery (H-J). APAC (red; C-D; F-G; I-J) was detected at the luminal side and in tunica media of vein (C-D), and around the suture sites (F-G). APAC was detected at the luminal side of the artery (I-J) outside the internal elastic lamina (arrow heads). Nuclei (blue; B; D-E; G-H; J) were detected with Hoechst stain against DNA. Asterisk = lumen. Scale bar corresponds to 20 μm.

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

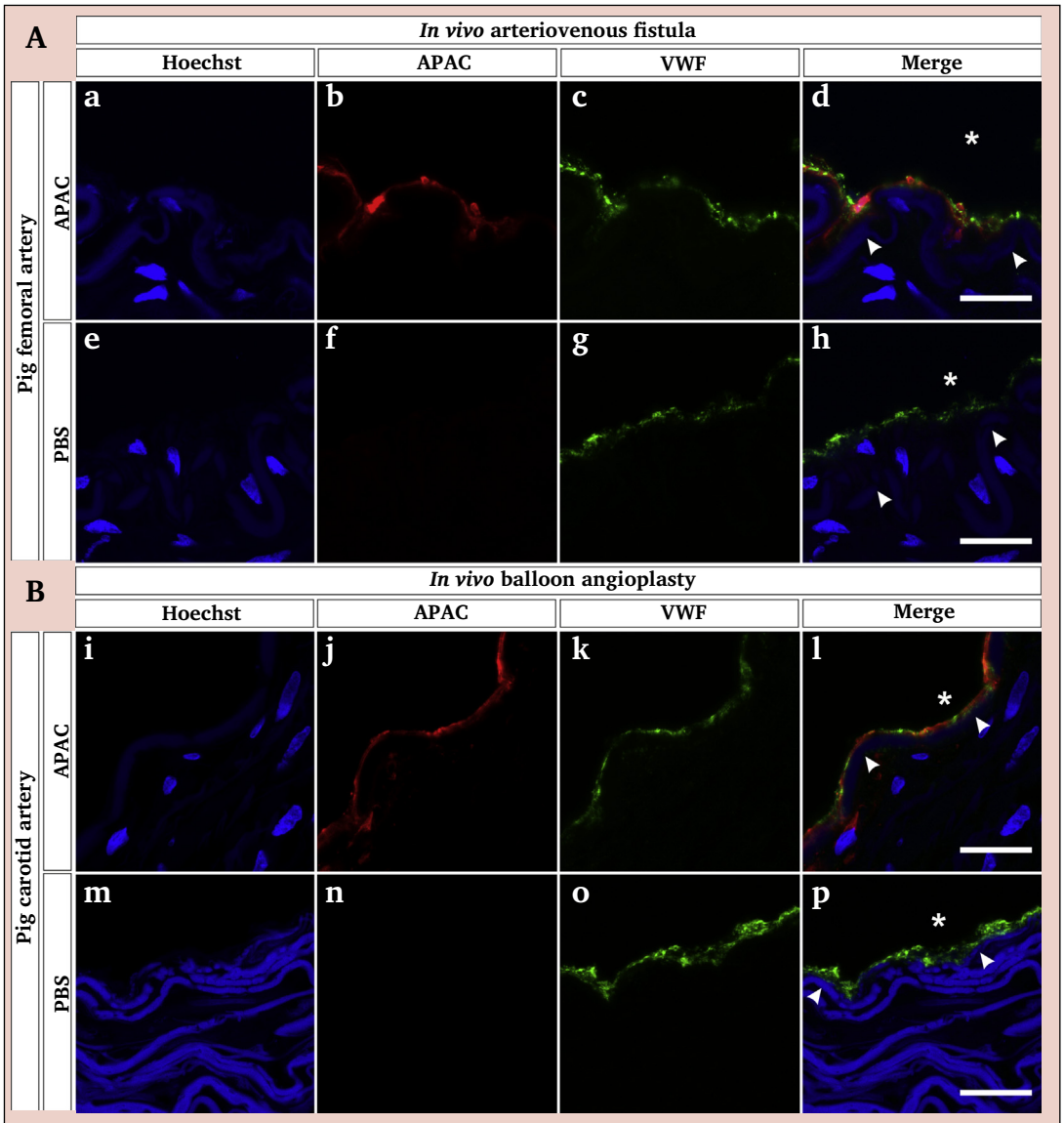

Figure 2. von Willebrand factor (vWF) and dual antiplatelet and anticoagulant (APAC) double staining of the luminal sides on the arterial side of femoral arteriovenous fistula (AVF, A) and balloon injured carotid artery walls (B) in pig. APAC or phosphate buffered saline (PBS) as control was incubated in AVF before opening the circulation and administrated through diffusion balloon catheter in carotid artery. Histological samples were immunostained for vWF (green; c–d; g–h; k–l; o–p) and APAC (red; b; d; f; h; j; l; n; p). Hoechst stain (blue) detected nuclei (a; d–e; h–i; l–m; p). In AVF and balloon injured carotid artery, APAC co-localised with vWF (d; l). In PBS controls, APAC signal was absent (f; h; n; p). Arrow heads = internal elastic lamina; Asterisk = lumen; Scale bar corresponds to 20 μm.

60% of the APAC signal co-localised with laminin (M1 = 0.60 ± 0.08; $n = 3$, and 0.64 ± 0.05, $n = 3$) (Fig. 3), showing APAC along the subendothelial basement membrane.

Limited APAC co-localisation with PECAM-1 and podocalyxin

To assess the binding of APAC to some patchy areas of intact endothelial cells, its co-localisation was studied with PECAM-1 and podocalyxin, both expressed by endothelial cells and platelets. Some luminal areas of intact endothelium were defined as linearly organised nucleated cells, being positive for PECAM-1 and/or podocalyxin. On the arterial side of the AVF, only 25% of the APAC signal co-localised with PECAM-1 (M1 = 0.25 ± 0.03; $n = 3$) (Fig. 4), when present. On the venous side, PECAM-1 was nearly absent, while strong penetration of APAC in the venous wall was observed (Fig. S5). Similarly, in the balloon angioplasty model in CAs, less than 25% of the APAC signal co-localised with PECAM-1 (M1 = 0.22 ± 0.04; $n = 3$) (Fig. 4). These values indicate that less than 25% of any of the signals co-localised with another signal. The M1 of APAC-vWF and APAC-PECAM-1 differed significantly in both AVF and balloon injury ($t(2) = 8.2$, $p < .01$ and 61.8, $p < .001$, respectively). In agreement with the results of PECAM-1, the presence of APAC was reduced also in podocalyxin positive areas (Fig. 5).

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

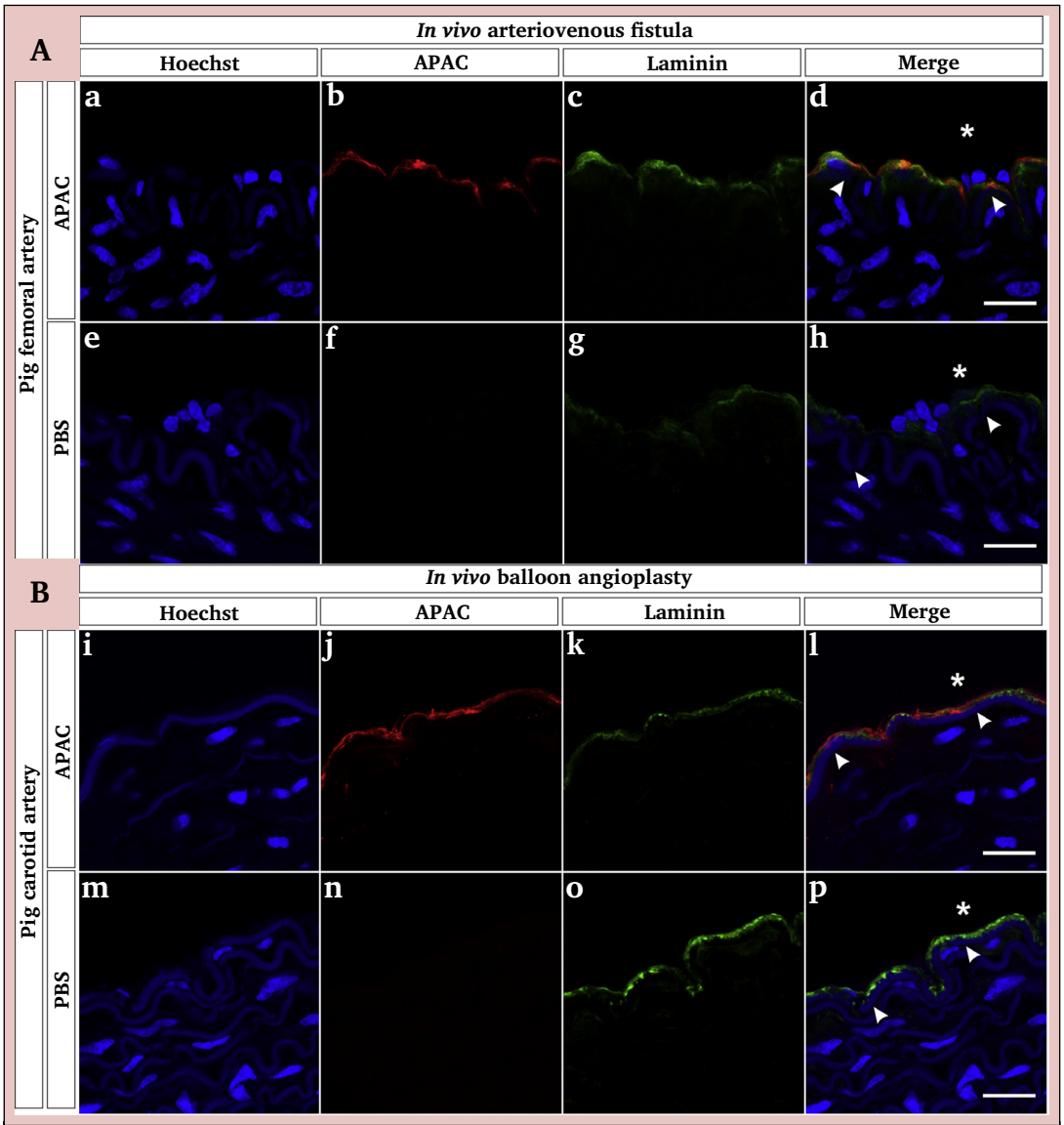

Figure 3. Laminin and dual antiplatelet and anticoagulant (APAC) double staining in arterial side of femoral arteriovenous fistula (AVF, A) and balloon denuded carotid artery (B) in pig. APAC or phosphate buffered saline (PBS) as control was incubated in AVF before opening the circulation and administrated through diffusion balloon catheter in carotid artery. Histological samples were stained for laminin ($\alpha1/\beta1/\gamma1$ chains, green; c–d; g–h; k–l; o–p) and APAC (red, b, d, f, h, j, l, n, p). Hoechst stain (blue) detected nuclei (a, d–e, h–i, l–m, p). APAC co-localised with laminin (d; l). In PBS controls, APAC signal was absent (f; h; n; p). Arrow heads = internal elastic lamina; Asterisk = lumen. Scale bar corresponds to 20 μm.

PECAM-1 and podocalyxin are both also expressed by platelets, but staining occurred in nucleated cells, and the antibody did not detect any signal in the white thrombi present locally in some areas of the AVFs.[16,17]

DISCUSSION

APAC, a mast cell derived HEP-PG mimic, is a semisynthetic conjugate of multiple heparin chains, which unlike UFH or any other current antithrombotic, has dual antiplatelet and anticoagulant properties. These have been characterised via several methods both *in vitro* and *in vivo*, leading to the name APAC (AntiPlatelet-AntiCoagulant).[7,8,10] Locally administered HEP-PG or APAC inhibits thrombus formation at the vascular injury site or collagen surface and by binding remains at the site of vascular injury.[8,10,17] To better delineate these specific vascular targeted interactions, the histological localisation of biotinylated APAC was studied at the injured vascular wall using various injury models, including the clinically relevant *in vivo* surgically created femoral AVF, IA and CA balloon injury, and *in vitro* mechanically denuded (scraped) FA. APAC multivalently targeted vascular injury sites by co-localizing with subendothelial structures, specifically with vWF and laminin. In contrast, APAC did not bind to vascular areas in which the endothelium seemed intact. The binding of APAC persisted at the site of injury even under very high flow conditions, and APAC was

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

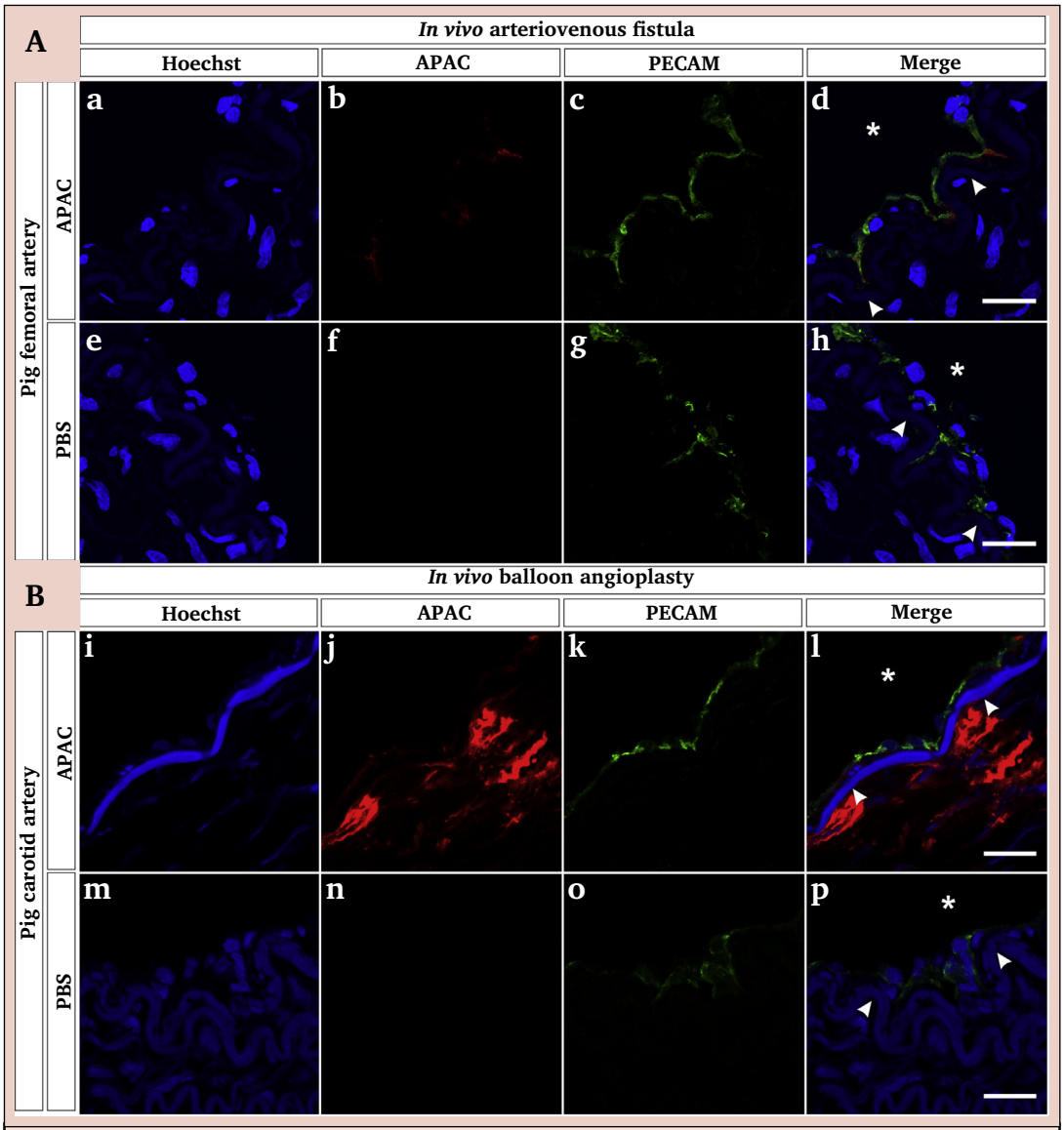

Figure 4. Platelet endothelial cell adhesion molecule 1 (PECAM-1) and dual antiplatelet and anticoagulant (APAC) double staining of femoral arteriovenous fistula (AVF) (A) and balloon injured carotid artery (B) in pig. APAC or phosphate buffered saline (PBS) as control was incubated in AVF before opening the circulation and administrated through diffusion balloon catheter in carotid artery. Histological samples were stained for platelet endothelial cell adhesion molecule 1 (PECAM-1; green; c-d; g-h; k-l; o-p) and for APAC (red, b, d, f, h, j, l, n, p). Hoechst stain (blue) detected nuclei (a, d-e, h-i, l-m, p). APAC did not co-localise with PECAM (d; l). In PBS controls, APAC signal was absent (f-h; n-p). Arrow heads = internal elastic lamina; Asterisk = lumen. Scale bar corresponds to 20 μm.

endovascularly administrated via the specific porous angioplasty balloon. As antiplatelet and anticoagulation effects are beneficial during endovascular interventions,[5,6] APAC could provide a sound option for preventing thrombotic complications at application sites.[10,11,18]

APAC directly interacts with the site of vascular injury in vitro *in the absence of blood flow*

During vascular injury the subendothelial basement membrane and the deeper layers of the vascular wall, rich in collagen, make contact with blood and initiate haemostasis, which may proceed to thrombosis. This results from the activation of coagulation and platelets via their glycoprotein receptors, which interact with vWF and collagen in the flowing blood, especially under high shear forces.[3]

In the *in vitro* model of mechanically denuded FA, without blood contact and endothelium, APAC provided the signal directly at the subendothelial areas in the absence of vWF. The present results show that direct binding of the highly negatively charged APAC to the positively charged extracellular matrix can also occur in the absence of blood or plasma components, and specifically, also in the absence of vWF *in situ*.[16]

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

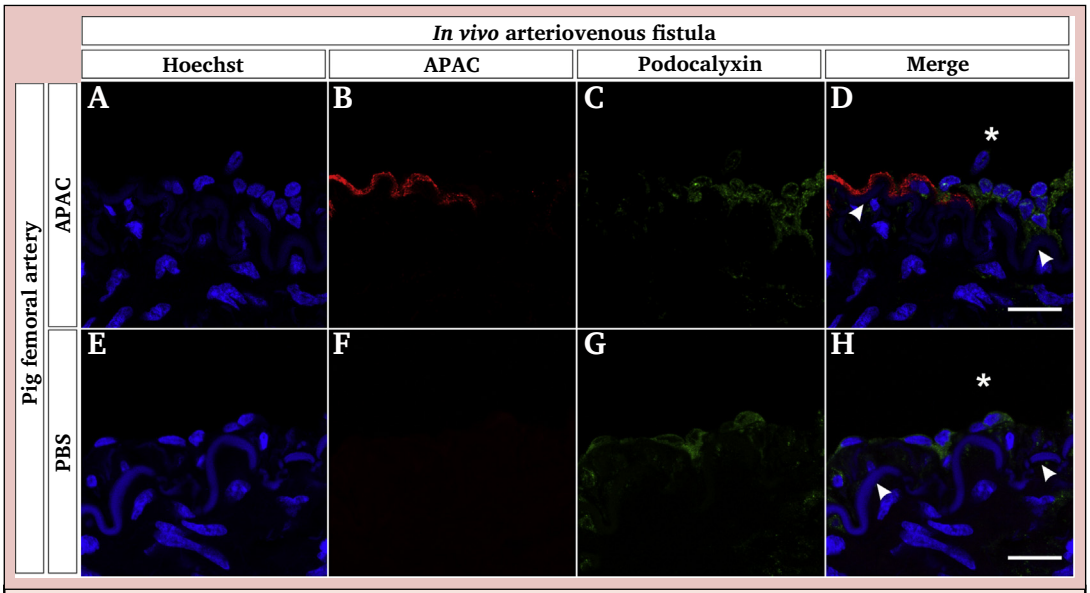

Figure 5. Podocalyxin and dual antiplatelet and anticoagulant (APAC) in femoral arteriovenous fistula (AVF). in pig. APAC or phosphate buffered saline (PBS) as control was incubated in AVF before opening the circulation and administrated through diffusion balloon catheter in carotid artery. Histological samples were stained for podocalyxin (green; C-D; G-H) and APAC (red; B; D; F; H). Hoechst stain (blue) detected nuclei (A; D-E; H). APAC signal was reduced in the podocalyxin positive areas (D). In PBS controls, APAC signal was absent (H). Arrow heads = internal elastic lamina; Asterisk = lumen. Scale bar corresponds to 20 μm.

APAC interacts in vivo *with the site of vascular injury in the presence of blood flow*

To study the influence of blood and its flow conditions on the interactive mechanisms of APAC and the vascular wall during vascular injury, two *in vivo* porcine models of severe IA and CA balloon injury were used. APAC was able to target and persist in the vascular wall after being subjected to the flushing effect of restored circulation, even resisting the AVF high flow conditions, indicating strong binding. APAC was administered locally to the vascular site using a porous angioplasty balloon. These techniques provide a practical rationale for APAC application and may also suggest other administration methods, that is covered stents or drug eluting balloons.

APAC binds in vivo *to balloon injury sites with absent endothelium co-localizing with vWF and laminin*

In the present balloon injury model, APAC mostly decorated the luminal surface without endothelium. The overlapping of APAC with vWF and laminin was 60—70% (M1). In addition to laminin, subendothelium contains collagens, that is collagen IV in the basal lamina, binding negatively charged heparins.[19] APAC overall interacts with the positively charged extracellular matrix components and plasma factors. At the balloon injury site, APAC infiltrated to the tunica media, but did not follow the elastic lamina, suggesting APAC's preference for extracellular matrix. Mast cell derived macromolecular HEP-PG, the model for APAC, attenuated platelet-collagen interactions in both soluble and immobilised form on collagen, and under high blood flow, the effect mediated by vWF.[7,8] Similar results were obtained recently on the role of vWF inhibition by APAC under blood perfusion over collagen and tissue factor, where APAC reduced platelet deposition at the arterial shear rate.[20]

APAC is retained in AVF and vascular injury under high flow conditions

High shear stress blood flow causes endothelial cell activation and enhances platelet adhesion through vWF, leading to thrombus formation.[21] In an earlier study on collagenous AVF loop grafts, APAC reduced platelet deposition, distal thrombus propagation, and fibrin accumulation, unlike UFH.[10] In this study, there was APAC binding to the AVF on the artery and vein luminal surfaces lacking endothelium, and APAC penetrated the vein. The stable co-localisation of APAC with vWF and laminin was similar in balloon injuries, suggesting that the interaction between APAC under high arterial flow conditions and vWF originates from sources other than endothelium, that is platelets and plasma. The outcome of vascular interventions, that is the AVF, is impaired by thrombophilic traits, and there is an urgent need for novel antithrombotic strategies to maintain AVF patency.[22]

APAC lacks systemic effects when administrated locally

UFH is used widely during vascular surgery, as systemic anticoagulation is needed when manipulating vessels,[5,6] but the patients are simultaneously at risk of bleeding. APAC attenuated haemostasis in the rat tail bleeding model at high systemic (IV) doses, but less than UFH at compatible APTT levels.[10] As in previous *in vivo* baboon studies of local small dose (2 mg) APAC,[10] no increase in systemic anticoagulation was detected in pigs, suggesting safety of APAC integration. As the majority of vascular patients will be on

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

aspirin or other platelet inhibitors peri-operatively co-administered with UFH, safety is paramount.

Study limitations

The follow up time of APAC interaction was short, only 30 min, and long term effects cannot be predicted. However, the current study was designed as the first step in the investigation of the early vascular vessel wall interaction with APAC. It aimed at piloting APAC's initial binding counterparts in the vascular wall at clinically relevant sites in vascular surgical approaches. The focus was on immediate APAC targeting on vascular structures, but there was no follow up on thrombus formation. In any case, it is shown that a short incubation time leads to firm binding of APAC during the period following open circulation, even under the high flow conditions of an AVF, providing a foundation for further studies. As the previous data in rats showed PET labelled APAC retention for at least 50 h at the application site,[10] a longer impact can be assumed also in these current models. Although the present histological data demonstrate the co-localisation of APAC with collagens, vWF, or laminin, the time course and longer follow up are warranted in these models. Also, because of species specific differences, the present results should be extrapolated with caution.

CONCLUSIONS

This study shows that APAC is easy to administer to target vascular injury and persists at the site under both normal and high arterial blood flow. APAC binding preferred vWF, laminin, and the basal lamina collagen, but vascular interaction of APAC was observed also in the absence of vWF. APAC did not target preserved endothelial areas. This localising feature may have potential antithrombotic implications for vascular interventions.

CONFLICT OF INTEREST

Karina Barreiro and Annukka Jouppila received a research grant from Aplagon Ltd., Helsinki, Finland. Riikka Tulamo and Anders Albäck have no conflicts of interest. Riitta Lassila is the CSO of Aplagon.

FUNDING

This work was supported by Aplagon Ltd., Helsinki, Finland.

ACKNOWLEDGEMENTS

We thank Olli Valtanen and Veikko Huusko for excellent animal care. Imaging was performed at the Biomedicum Imaging Unit of the University of Helsinki, Finland.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejvs.2019.05.019.

REFERENCES

1 Yau JW, Teoh H, Verma S. Endothelial cell control of thrombosis. *BMC Cardiovasc Dis* 2015;**15**:130.
2 Jackson SP, Nesbitt WS, Westein E. Dynamics of platelet thrombus formation. *J Thromb Haemost* 2009;**7**:17–20.
3 Ruggeri ZM, Mendolicchio GL. Interaction of von Willebrand factor with platelets and the vessel wall. *Hamostaseologie* 2015;**35**:211–24.
4 Mammadova-Bach E, Ollivier V, Loyau S, Schaff M, Dumont B, Favier R, et al. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood* 2015;**126**:683–91.
5 Schmidli J, Widmer MK, Basile C, de Donato G, Gallieni M, Gibbons CP, et al. Editor's choice - vascular access: 2018 clinical practice guidelines of the European society for vascular surgery (ESVS). *Eur J Vasc Endovasc Surg* 2018;**55**:757–818.
6 Naylor AR, Ricco JB, de Borst GJ, Debus S, de Haro J, Halliday A, et al. Editor's choice - management of atherosclerotic carotid and vertebral artery disease: 2017 clinical practice guidelines of the European society for vascular surgery (ESVS). *Eur J Vasc Endovasc Surg* 2018;**55**:3–81.
7 Lassila R, Lindstedt K, Kovanen PT. Native macromolecular heparin proteoglycans exocytosed from stimulated rat serosal mast cells strongly inhibit platelet-collagen interactions. *Arterioscler Thromb Vasc Biol* 1997;**17**:3578–87.
8 Kauhanen P, Kovanen PT, Lassila R. Coimmobilized native macromolecular heparin proteoglycans strongly inhibit platelet-collagen interactions in flowing blood. *Arterioscler Thromb Vasc Biol* 2000;**20**:E113–9.
9 McFadyen JD, Jackson SP. Differentiating haemostasis from thrombosis for therapeutic benefit. *Thromb Haemost* 2013;**110**:859–67.
10 Lassila R, Jouppila A. Mast cell-derived heparin proteoglycans as a model for a local antithrombotic. *Semin Thromb Hemost* 2014;**40**:837–44.
11 Tuuminen R, Jouppila A, Salvail D, Laurent CE, Benoit MC, Syrjala S, et al. Dual antiplatelet and anticoagulant APAC prevents experimental ischemia-reperfusion-induced acute kidney injury. *Clin Exp Nephrol* 2017;**21**:436–45.
12 Steele PM, Chesebro JH, Stanson AW, Holmes Jr DR, Dewanjee MK, Badimon L, et al. Balloon angioplasty. Natural history of the pathophysiological response to injury in a pig model. *Circ Res* 1985;**57**:105–12.
13 Wang Y, Krishnamoorthy M, Banerjee R, Zhang J, Rudich S, Holland C, et al. Venous stenosis in a pig arteriovenous fistula model–anatomy, mechanisms and cellular phenotypes. *Nephrol Dial Transpl* 2008;**23**:525–33.
14 Rotmans JI, Velema E, Verhagen HJ, Blankensteijn JD, Kastelein JJ, de Kleijn DP, et al. Rapid, arteriovenous graft failure due to intimal hyperplasia: a porcine, bilateral, carotid arteriovenous graft model. *J Surg Res* 2003;**113**:161–71.
15 Manders EMM, Verbeek FJ, Aten JA. Measurement of co-localization of objects in dual-colour confocal images. *J Microsc* 1993;**169**:375–82.
16 Janmey PA, Slochower DR, Wang YH, Wen Q, Cebers A. Polyelectrolyte properties of filamentous biopolymers and their consequences in biological fluids. *Soft Matter* 2014;**10**:1439–49.
17 Olsson E, Asko-Seljavaara S, Lassila R. Topically administered macromolecular heparin proteoglycans inhibit thrombus growth in microvascular anastomoses. *Thromb Haemost* 2002;**87**:245–51.
18 Favaloro EJ. A short history of thrombosis and hemostasis: part II (40th year celebratory issue). *Semin Thromb Hemost* 2014;**40**:826–30.
19 Parkin JD, San Antonio JD, Pedchenko V, Hudson B, Jensen ST, Savige J. Mapping structural landmarks, ligand binding sites, and missense mutations to the collagen IV heterotrimers predicts major functional domains, novel interactions, and variation in

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019

phenotypes in inherited diseases affecting basement membranes. *Hum Mutat* 2011;**32**:127–43.

20 Chen J, Verni CC, Jouppila A, Lassila R, Diamond SL. Dual antiplatelet and anticoagulant (APAC) heparin proteoglycan mimetic with shear-dependent effects on platelet-collagen binding and thrombin generation. *Thromb Res* 2018;**169**:143–51.

21 Cosemans JM, Angelillo-Scherrer A, Mattheij NJ, Heemskerk JW. The effects of arterial flow on platelet activation, thrombus growth, and stabilization. *Cardiovasc Res* 2013;**99**:342–52.

22 Goli RR, Contractor MM, Nathan A, Tuteja S, Kobayashi T, Giri J. Antiplatelet therapy for secondary prevention of vascular disease complications. *Curr Atheroscler Rep* 2017;**19**:56.

Please cite this article as: Barreiro KA et al., Novel Locally Acting Dual Antiplatelet and Anticoagulant (APAC) Targets Multiple Sites of Vascular Injury in an Experimental Porcine Model, European Journal of Vascular and Endovascular Surgery, https://doi.org/10.1016/j.ejvs.2019.05.019