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Low dose Adenoviral Vammin gene transfer induces myocardial angiogenesis and increases left ventricular ejection fraction in ischemic porcine heart

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This preliminary study investigated if VEGFR-2 selective adenoviral Vammin (AdVammin) gene therapy could induce angiogenesis and increase perfusion in the healthy porcine myocardium. Also, we determined using a clinically relevant large animal model if AdVammin gene therapy could improve the function of a chronically ischemic heart. Low doses of AdVammin (dose range 2×10^9–2×10^{10} vp) gene transfers were performed into the porcine myocardium using an endovascular injection catheter. AdCMV was used as a control. The porcine model of chronic myocardial ischemia was used in the ischemic studies. The AdVammin enlarged the mean capillary area and stimulated pericyte coverage in the target area 6 days after the gene transfers. Using positron emission tomography ^{15}O-radiowater imaging, we demonstrated that AdVammin gene therapy increased perfusion in healthy myocardium at rest. AdVammin treatment also increased ejection fraction at stress in the ischemic heart, as detected using left ventricular cine angiography. In addition, we demonstrated successful in vivo imaging of enhanced angiogenesis using [^{68}Ga]NODAGA-RGD peptide. However, AdVammin also increased tissue permeability and was associated with significant pericardial fluid accumulation, limiting AdVammin's therapeutic potential and emphasizing the importance of correct dosage.

Ischemic heart disease remains a significant cause of mortality and morbidity worldwide[1,2]. Unfortunately, many patients do not benefit or are unsuitable for conventional coronary artery disease (CAD) treatments, such as coronary angioplasty, stenting, and coronary artery bypass grafting (CABG)[3,4]. Thus, there is an urgent need for minimally invasive treatments to restore perfusion to the ischemic myocardium efficiently and safely in this group of refractory angina patients. Proangiogenic gene therapy using vascular endothelial growth factor (VEGF) family members is a potential new approach for the treatment of these patients. However, previous VEGF-A gene therapy has not produced beneficial clinical effects and new approaches are needed to test proangiogenic gene therapies in preclinical and clinical studies[567]..

VEGFs include vertebrate VEGFs A-D, viral genome-derived VEGF-E, placental growth factor (PlGF) and VEGF-F[8]. Previously, mainly VEGF-A binding to VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2), VEGF-B binding to VEGFR-1, and VEGF-D and VEGF-C binding to VEGFR-2 and VEGFR-3 have been used in proangiogenic gene therapy[67891011121314]. Besides binding to the VEGFRs, VEGFs also bind with different affinities to their co-receptors, neuropilins 1 and 2 (Nrp-1 and Nrp-2) and heparan sulfate proteoglycans (HSPGs), which are crucial modulators of intracellular signalling and essential for angiogenesis[8,15]. VEGFR-2 is considered to mediate most of the angiogenic effects of the VEGFs, whereas VEGFR-1 is linked to the inflammatory responses in addition to its angiogenetic effects[16,17], and VEGFR-3 mediates mainly lymphangiogenesis[18]. Theoretically, gene therapy using VEGFR-2 selective ligands could result in beneficial angiogenic effects with lower doses and thus with minimal adverse effects, such as inflammation.

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Vammin is one of the VEGF-Fs, a family of VEGFs isolated from Viper venom[19,20]. VEGF-Fs bind specifically to VEGFR-2 with no affinity to VEGFR-1 or −3. Vammin also binds to Nrps and HSPGs[21]. Due to this unique binding profile, Vammin is a potential candidate as a proangiogenic agent. Furthermore, gene transfer using adenoviral (Ad) Vammin has increased perfusion, capillary size and vascular permeability in rabbit skeletal muscles[21,22]. In the current preliminary study, we evaluated the therapeutical potential and safety of AdVammin gene therapy in healthy and ischemic porcine myocardium in the exploratory-like study setting.

Methods

Animal experiments

Domestic female pigs (25 kg) were used for the study. For sedation, an intramuscular injection of 1.5 mL atropine (1 mg/mL; Leiras, Helsinki, Finland) and 6 mL azaperone (Stresnil® 40 mg/mL; Janssen Pharmaceutica N.V., Beerse, Belgium) were given. For anesthesia, an intravenous propofol infusion (Propofol-Lipuro 20 mg/mL; B. Braun, Melsungen, Germany) at 15 mg/kg/h and fentanyl (Fentanyl™ 50 µg/mL; Janssen-Cilag, Espoo, Finland) at 10 µg/kg/h was used. Intravenous potassium chloride was used for euthanasia. The Finnish National Animal Experiment Board and the Animal Experiment Board of the University of Finland approved all animal experiments. All animal experiments were in compliance with the ARRIVE guidelines.

The effects of AdVammin gene transfer with different doses were first assessed in the healthy porcine myocardium to determine optimal dosing. Second, using the optimal dose, a separate set of animals was imaged using positron emission tomography (PET) for perfusion changes and angiogenesis. Third, effects with the optimal dose were studied in the ischemic heart using the bottleneck stent model of chronic myocardial ischemia[19]. Animals in the control groups received an empty Ad with the same cytomegalovirus promoter (AdCMV). Different animal groups and their respective endpoints are presented in Table 1..

Gene transfers

Gene transfers were done percutaneously into the anterior and lateral walls of the left ventricle using Noga® Myostar (Biosense Webster, a Johnson & Johnson company, Diamond Bar, CA, USA) injection catheter equipped with the 27G needle. For ischemic pigs, an electroanatomical map of the left ventricle was acquired, and according to this map, injections were administered to the hypokinetic but still viable areas[12,23]. AdVammin gene transfers to the normoxic pigs were done with the doses of 2×10^9 viral particles (vp), 1×10^{10} vp, and 2×10^{10} vp as ten intramyocardial injections (200 µl per injection). Ischemic pigs received an AdVammin dose of 1×10^{10} vp (10 injections, 200 µl each). Animals in the control groups received an AdCMV dose of 1×10^{10} or 2×10^{10}vp (10 injections, 200 µl each). Replication-deficient human serotype 5 adenoviruses produced under GMP conditions were used for the studies[12,24]. Different doses and groups are presented in Table 1..

Coronary angiography, left ventricular cine angiography and echocardiography

Macroscopically visible angiogenesis in the myocardium was assessed with coronary angiography (Innova® 3100^{IQ}; GE Healthcare, USA). Standard Amplatz right 1 (AR-1) and AR-2 type diagnostic catheters were used to image the right and left coronary arteries. An iodine-based contrast agent (Iomeron®, 350 mg/ml, Bracco) was used for the visualization. Left ventricular cine angiography (LV-CINE) was used to measure ejection fraction (EF) and cardiac output (CO) to assess the left ventricular function of the ischemic animals. A pigtail-type catheter was used for the catheterization of the left ventricle. LV-CINE was performed during rest, and pharmacological stress induced by i.v. Dobutamine (Dobumin Hameln 12,5 mg/mL; Hameln Pharma Plus Gmbh, Hameln, Germany) infusion starting at 10 µg/kg/min and increasing the dose if needed until the heart rate of 160 bpm was reached. The CO was calculated as the product of stroke volume and heart rate. EF and CO were determined from LV-CINE images using Simpson's method. Echocardiography (Acuson Sequoia 512, Siemens AG, Germany) was performed to visualize possible pericardial fluid accumulation. Coronary angiography, LV-CINE and echocardiography were performed right before and six days after the gene transfers.

	Treatment	N	Timepoints	Endpoints
Healthy myocardium, group 1	AdCMV 2×10^{10} vp	5	6 days	AG, ECHO, IHC, MMA
	AdVammin 2×10^{9} vp	5	6 days	AG, ECHO, IHC, MMA
	AdVammin 1×10^{10} vp	4	6 days	AG, ECHO, IHC, MMA
	AdVammin 2×10^{10} vp	5	6 days	AG, ECHO, IHC, MMA
Healthy myocardium, group 2	AdCMV 1×10^{10} vp	4	6 days	PET-perfusion, PET-RGD
	AdVammin 1×10^{10} vp	4	6 days	PET-perfusion, PET-RGD
Chronic ischemia, group 3	AdCMV 1×10^{10} vp	5*	6 days	LVEF, CO
	AdVammin 1×10^{10} vp	5*	6 days	LVEF, CO
Chronic ischemia, group 3	AdCMV 1×10^{10} vp	5**	28 days	Infarct size
	AdVammin 1×10^{10} vp	5**	28 days	Infarct size

Table 1.. Different treatment groups and endpoints. Abbreviations: angiography (AG), left ventricular ejection fraction (LVEF), cardiac output (CO), echocardiography (ECHO), immunohistochemistry (IHC), Modified Miles Assay (MMA), positron emission tomography (PET). *n = 4 for LVEF and CO measurements. ** Same animals as in LVEF and CO measurements.

Ischemia operations

Ischemia operations were performed two weeks before the gene transfers using a bottleneck stent model[9,25]. Briefly, a bottleneck-shaped polytetrafluoroethylene tube was mounted on top of an 8 mm long bare-metal stent (Coroflex Blue, B.Braun), and the construct was deployed into the proximal part of the left anterior descending coronary artery (LAD). A successful deployment was confirmed using a contrast agent and fluoroscopy. Animals received 2.5 mg of bisoprolol (Bisoprolol, Ratiopharm) and 200 mg of amiodarone (Cordarone, Sanofi-Aventis) daily, starting one week before the ischemia operation. Loading doses of 300 mg acetylic salicylic acid (ASA, Ratiopharm) and 300 mg of clopidogrel (Plavix, Sanofi-Aventis) were administered the day before the ischemia operation, and these medications continued after the ischemia operations for two days with daily doses of 100 mg and 75 mg, respectively. At the beginning of the ischemia operation, animals received 100 mg of lidocaine i.v. (Lidocaine 10 mg/mL; Orion Pharma, Espoo, Finland) and 2.5 mL $MgSO_4$ i.v. (Addex-magnesium sulfate 246 mg/mL; Fresenius Kabi, Uppsala, Sweden) to prevent ventricular arrhythmias and 30 mg of enoxaparin i.v. (Klexane®; Sanofi-aventis, Helsinki, Finland) to prevent thrombosis. Also, 30 mg of enoxaparin was given s.c. right after the ischemia operation and during the two following days. The ischemic pain was managed by giving 0.3 mg doses of buprenorphine (Temgesic, Reckitt&Colman Products) i.m. immediately after the operation and during the following days if signs of ischemic pain were detected.

PET-imaging of perfusion and angiogenesis

Positron emission tomography (PET) imaging with ^{15}O-radiolabeled water (805 ± 87 MBq) was used to determine regional myocardial perfusion at rest and during pharmacological stress six days after the gene transfer as previously described[9,10,26]. Pharmacological stress was induced with i.v. Adenosine at the rate of 200 µg/kg/min (Adenosine Life Medical, Life Medical Sweden AB, Stocksund, Sweden) combined with phenylephrine 5 µg/kg/min (Fenylefrin Abcur, Abcur AB, Helsingborg, Sweden) i.v. starting 2 min before imaging and continuing throughout the stress study to induce myocardial hyperemia. The phenylephrine was used to limit systemic hypotension due to the high adenosine dose, as the clinically used adenosine dose of 140 µg/kg/min is not high enough to induce maximal hyperemia in pigs. PET studies were performed using the ECAT EXACT HR + scanner (Siemens-CTI, Knoxville, TN, USA). The acquisition frames 14 × 5 s, 3 × 10 s, 3 × 20 s, and 4 × 30 s (total duration 4 min 40 s) were acquired. The results were calculated from the segments corresponding to the perfusion areas provided by the LAD and RCA, representing the treatment and control areas, respectively. Relative perfusion in the treatment area was calculated by dividing the absolute perfusion in the treatment area by the perfusion in the control areas.

To study myocardial angiogenesis in vivo [^{68}Ga]NODAGA-RGD PET was performed six days after the gene transfer as described previously[27]. Shortly, [^{68} Ga]NODAGA-RGD (353 ± 47 MBq) was injected via the ear vein. After the PET scanning, the hearts were excised, myocardial samples from the gene transfer site and a remote-control area were collected, and [^{68} Ga]NODAGA-RGD uptake was measured by autoradiography. Tissue morphology was studied by Hematoxylin–eosin staining, and blood vessel endothelium was detected with immunohistochemical staining using a CD31 antibody (Thermo Scientific RB-10333).

Modified Miles Assay

Modified Miles Assay was used to measure the effect of AdVammin on vascular permeability as described[28]. An intravenous 30 mg/kg injection of Evans Blue dye (Sigma) was administered 30 min before sacrification through the ear vein to the animals. Tissue samples from the gene transfer and control areas were collected, and their weight was measured. The extravasated dye from the tissues was diluted in 4 mL of formamide by incubating the samples at 68 °C for 48 h. The absorbance of the samples at 630 nm was determined using a plate reader (CLARIOstar plate reader, BMG Labtech, Ortenberg, Germany), and the relative amount of the extravasated dye at the gene transfer area in comparison to the control area was calculated.

Histological analysis and immunohistochemistry

For immunohistochemical stainings, tissue samples were collected from the injection sites and control areas of the left ventricle and other organs (lung, liver, spleen, kidney, and ovaries) for safety purposes. The hearts were perfusion fixed using 1500 ml 1% paraformaldehyde in citrate buffer, after which they were immersed in 4% paraformaldehyde for 4 h at room temperature and then incubated in 15% sucrose for 24 h at 4 °C. Myocardial samples from ischemic animals were not perfused with paraformaldehyde but immersion fixed in 4% paraformaldehyde for 24 h after tetrazolium chloride staining. After the fixation, tissue samples were dehydrated, embedded in paraffin and cut into 7 µm thick sections. CD31-immunostain using (PECAM-1 antibody (dilution 1:100, AF806, R&D) was used to measure the size and the number of capillaries. The presence of pericytes surrounding the endothelial cells was determined using a CD31 and smooth-muscle-actin (αSMA, Dako Mouse Anti Human Smooth Muscle Actin Clone 1A4) double-immunostaining.

Infarct area

The amount of infarcted myocardium was determined 28 days after the gene transfer. Briefly, the left ventricles of the ischemic animals were cut into 10 mm thick short-axis slices and incubated in tetrazolium chloride before fixation to stain viable myocardium. The infarct area was calculated from photographs taken from five short-axis sections of the heart using ImageJ software (https://imagej.nih.gov/ij/) as previously described[25].

GO analysis

For gene ontology (GO) analysis, we used our previously published GSE68535 bulk RNA-sequencing data (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68535) in which HUVEC cells were transduced

with AdVammin or AdCMV control vector for 48 h (n = 3 replicates)[21]. The following thresholds were used for analysis: FDR < 0.05, rpkm > 0.5 and log fold change > 1.0 and < −1.0. EnrichR web server was used for GO analysis (http://geneontology.org/).

Statistical analyses

All data is reported as mean ± SD. When two groups are compared, students' T-test with two-tailed p-value and significance at $p < 0.05$ is used. When comparing more than two groups, an ordinary one-way ANOVA with Tukey's multiple comparisons tests is used with a significance at $p < 0.05$. In figures, statistical significance is marked by "ns" or asterisks (ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$).

Results

AdVammin caused fluoroscopically detectable extravasation of contrast agent in healthy porcine myocardium

During the coronary angiography, the injection sites of AdVammin showed extravasation of the contrast agent six days after the gene transfer in all AdVammin groups, whereas the injection sites could not be visualized in the AdCMV control group (Fig. 1A–E and J). Naked eyes could clearly detect the exact injection sites of the AdVammin in the anterior wall of the left ventricle (Fig. 1B–E). There was a clear dose dependence in the extravasation of the contrast agent, and this effect was the most significant in the two highest AdVammin doses. Also, pericardial fluid accumulation could be visualized in the fluoroscopy and cardiac ultrasound (Fig. 1C-E and G–J). No pericardial fluid accumulation was detected in the AdCMV control group (Fig. 1A and F).

AdVammin increased vascular permeability in the treatment area

After intravenous Evans Blue dye administration, the gene transfer areas in the AdVammin groups could be visualized macroscopically from the removed hearts (Fig. 2A–D) and can also be visualized from the cross-sectional short-axis slices of the myocardium (Fig. 2E–H). The stained areas correspond well with the injection sites. The relative permeability in the gene transfer area as quantified using modified Miles assay was 1.39 ± 0.45, 2.35 ± 0.60, 2.99 ± 0.60, and 3.34 ± 0.63 for groups AdCMV 2×10^{10} vp, AdVammin 2×10^{9} vp, AdVammin 1×10^{10} vp, and AdVammin 2×10^{10} vp, respectively (Fig. 2I). The changes were statistically significant for the AdVammin doses 1×10^{10} vp and 2×10^{10} vp compared to the AdCMV 2×10^{10} control group (Fig. 2I). Accordingly, RNA-sequencing data from endothelial cells transduced with AdVammin (GSE98060) showed

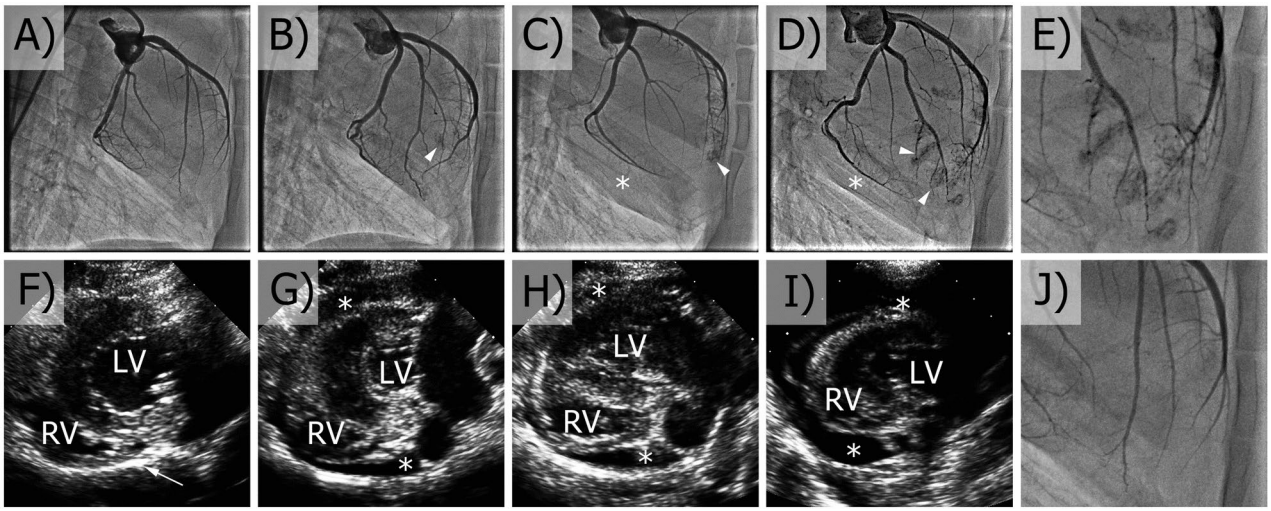

Fig. 1. AdVammin gene transfer induced fluoroscopically visible injection sites in porcine myocardium six days after the gene transfer. (**A**–**D**) shows representative angiograms of the left coronary artery six days after the gene transfer, taken from RAO 90 angle, for groups AdCMV 2×10^{10} vp (**A**), AdVammin 2×10^{9} vp (**B**), 1×10^{10} vp (**C**), and 2×10^{10} vp (**D**). In the AdCMV group, no injection sites could be visualized (**A**). However, in the AdVammin treated animals, the injection sites (marked with arrowheads) are visible (**B**–**D**). The effect was the most visible in the AdVammin 2×10^{10} vp dose (**D**). To highlight the effect, zoomed pictures of the angiograms for groups AdCMV 2×10^{10} vp and AdVammin 2×10^{10} vp are shown in J and E, respectively. In higher AdVammin doses, pericardial fluid accumulation six days after the gene transfer can be visualized in the fluoroscopy images (asterisk, **C** and **D**). The pericardial fluid accumulation for groups AdVammin 2×10^{9} vp (**G**), 1×10^{10} vp (H), and 2×10^{10} vp (**I**) could also be visualized in echocardiography (asterisk, **G**–**I**). No pericardial fluid was seen in the AdCMV control group (**F**). The left and right ventricle of the heart is marked in the echocardiography images by LV and RV, respectively. The echocardiograms (**F**–**I**) represent the parasternal short-axis view of the heart. However, the views are suboptimal due to the limited acoustic window when imaging pigs with very narrow intercostal spaces.

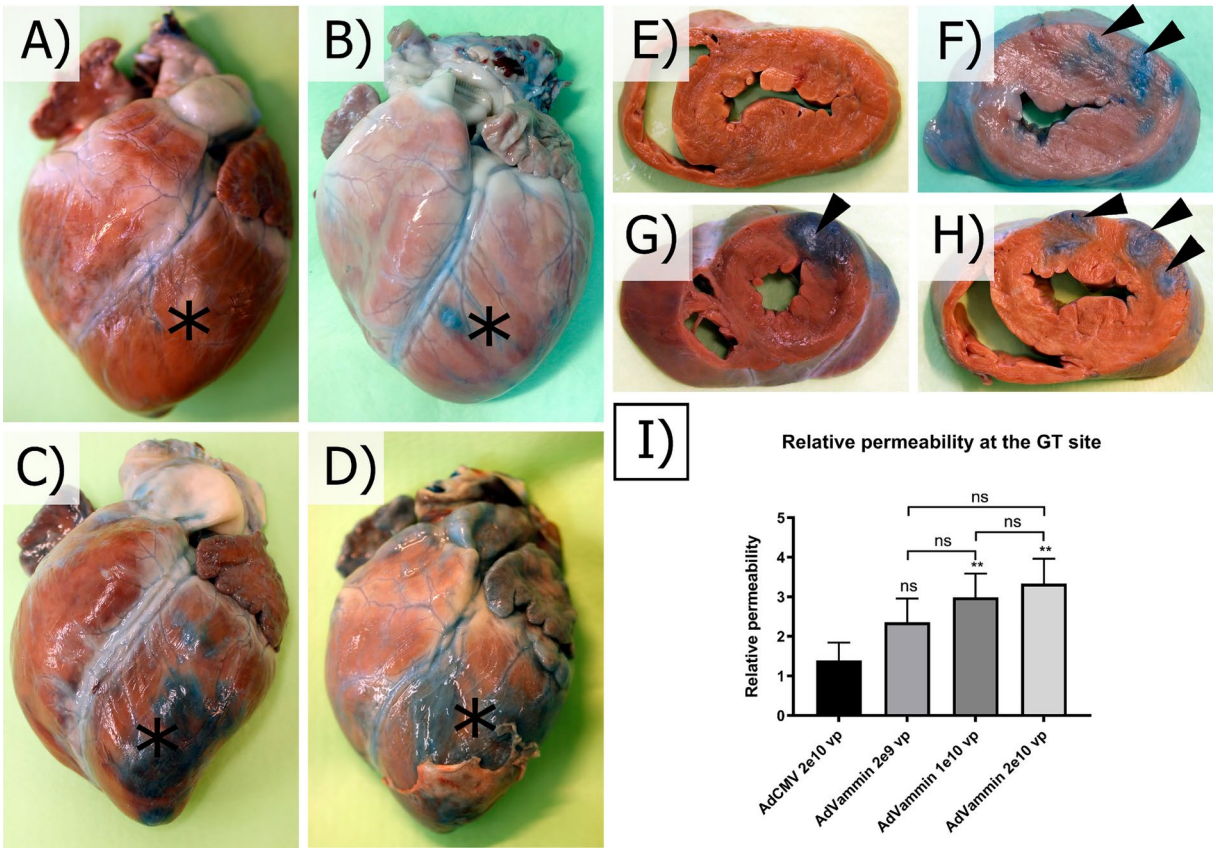

Fig. 2. AdVammin increased the vascular permeability six days after the gene transfer in the modified Miles Assay. Representative picture of the excised hearts (**A**-**D**) and cross-sections (**E**–**H**) of the same heart for groups AdCMV 2×10^{10} vp (**A** and **E**), AdVammin 2×10^{9} vp (**B** and **F**), AdVammin 1×10^{10} vp (**C** and **G**) and AdVammin 2×10^{10} vp (**D** and **H**) are shown in **A**–**H**, with the target areas shown using black asterisks and arrowheads. The quantified relative vascular permeability in the gene transfer target site is represented in graph I. Statistical significance is marked by "ns" or asterisks (ns = $p > 0.05$ and ** = $p \leq 0.01$). Increased vascular permeability was statistically significant in AdVammin 1×10^{10} vp and 2×10^{10} vp groups when compared to the AdCMV control group.

GO	Term	Genes
0002040 0045765	Sprouting Angiogenesis Regulation of Angiogenesis	**NR4A1**;**ESM1**;**BMPER**;PGF ECM1;SPINK5;GAB1;HMGA2; BRCA1;VASH1;PGF;RGCC;BMPER;FGF18; CYP1B1;**ANGPTL4**;**CD34**
0043117	Positive regulation of vascular permeability	**PDE2A**
0030155	Regulation of cell adhesion	TNFSF18;**CXCL12**;**PODXL**;TESC; KIF14;CXCR4;C2CD4B;**GPR4**; **PIK3CG**
0030198	Extracellular matrix organization	POSTN;COL14A1;MMP1;LUM;ELNSPINK5;CTSV;NID2;MMP10; COL1A1;ADAMTS15;SCUBE3; CREB3L1;SMOC1;P4HA3;HAS3; CYP1B1;COL21A1;COL9A3;ITGA6;A2M
0048660	Regulation of smooth muscle cell proliferation	NR4A3;ELN

Table 2. Selected GO pathways regulated by AdVammin in endothelial cells affecting vascular permeability or angiogenesis-related events.

regulation of genes associated with vascular permeability, such as NR4A1, ESM1, BMPER, ANGPTL4 and PDE2A (Table 2).

AdVammin promoted angiogenesis in a dose-dependent manner and resulted in large capillary vessels with pericyte coverage

The AdVammin gene transfer to the healthy myocardium increased the mean capillary area at the treatment area dose-dependently six days after the gene transfer. The average capillary area in the AdCMV control group was 11.47 ± 2.01 μm^2, whereas in the AdVammin treated animals, it was 60.68 ± 5.44 μm^2, 94.05 ± 12.63 μm^2 and $96{,}06 \pm 29.85$ μm^2 with the doses of 2×10^{9} vp, 1×10^{10} vp and 2×10^{10} vp, respectively (Fig. 3A–D and I).

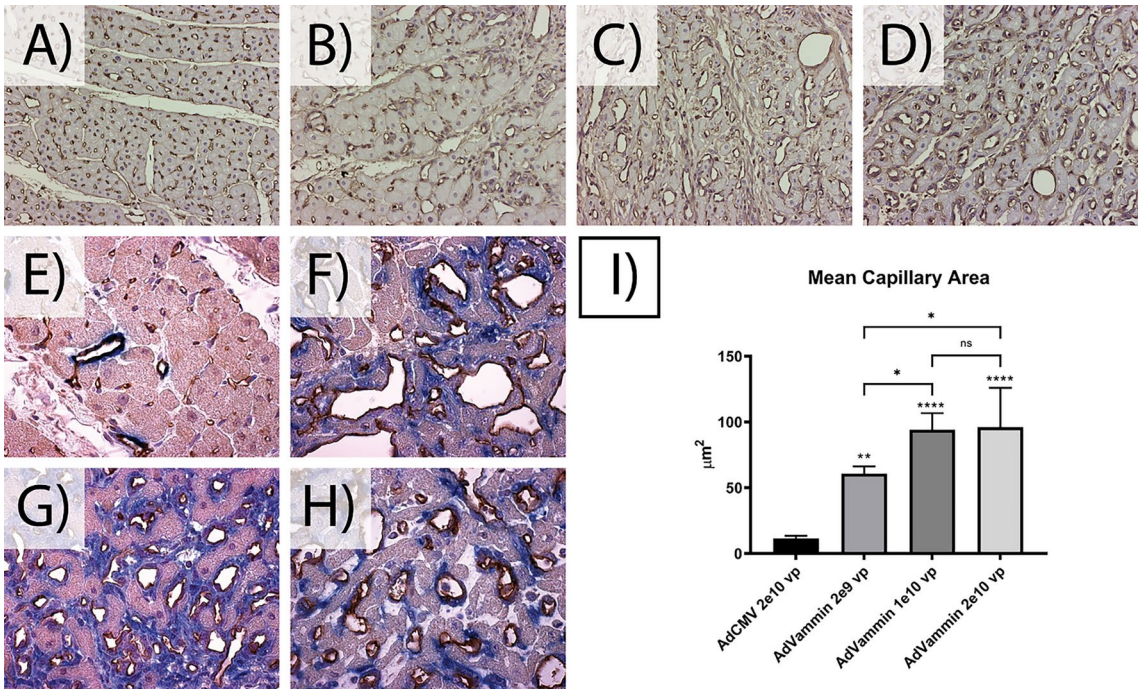

Fig. 3. AdVammin gene transfer increased the mean capillary area in the porcine myocardium six days after the gene transfer. CD31-immunostained sections from the gene transfer area at 20 × magnification, with endothelial cells stained brown, for the AdCMV 2×10^{10} vp, AdVammin 2×10^{9} vp, AdVammin 1×10^{10} vp and AdVammin 2×10^{10} vp groups are shown in A–D, respectively. The gene transfer with AdVammin significantly increased the mean capillary area in all dose groups. There was a significant difference in capillary size between the lowest AdVammin dosage and the higher dosages (I). Statistical significance is marked by "ns" or asterisks (ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$). Also, six days after the AdVammin gene transfer, the enlarged vessels showed clear pericyte coverage, as seen from the cd31-αSMA-immunostaining, with cd31 stained brown and αSMA stained blue (F–H). Representative pictures of CD31-αSMA-immunostained sections for groups AdCMV 2×10^{10} vp, AdVammin 2×10^{9} vp, AdVammin 1×10^{10} vp, and AdVammin 2×10^{10} vp taken at 50 × magnification are shown in E–H, respectively.

All these changes were statistically significant except the difference between AdVammin doses of 1×10^{10} vp and 2×10^{10} vp (Fig. 3. I). In the CD31-αSMA-immunostaining the enlarged capillaries in AdVammin-treated animals had clear αSMA-positive pericyte coverage compared with the AdCMV group (Fig. 3E–H). Also, RNA-sequencing data from endothelial cells transduced with AdVammin (GSE98060) showed regulation of genes associated with sprouting angiogenesis and smooth muscle cell proliferation (Table 2).

The uptake of [^{68} Ga]NODAGA-RGD peptide is increased in the area of enhanced angiogenesis

In the AdCMV control group, myocardial morphology and blood vessel endothelial stainings were similar in the gene transfer site and the control areas (Fig. 4). In the AdVammin group, the myocardium in the transfer site showed a local angiogenic response in all pigs, as shown in Fig. 4. The myocardial area stained with CD31 was 1.6 ± 0.39 folds higher in the gene transfer site than in the control area after AdVammin treatment, whereas there was no difference in the AdCMV group as the ratio was 1.1 ± 0.26 (Fig. 4 D–F, K).

Analysis of PET images showed an increased uptake of [^{68} Ga]NODAGA-RGD in the AdVammin gene transfer sites compared to the control area, whereas there was no difference between sites in the AdCMV group. The ratios of [^{68} Ga]NODAGA-RGD uptake in the gene transfer site and control area in the AdVammin and AdCMV groups were 1.1 ± 0.05 and 0.88 ± 0.16 ($p = 0.04$), respectively. Autoradiography confirmed a local increase in the myocardial uptake of [^{68} Ga]NODAGA-RGD in the gene transfer site in the AdVammin group. Using autoradiography, the [^{68} Ga]NODAGA-RGD uptake ratio between the target area and the control area was 1.7 ± 0.45 in the AdVammin group and 1.0 ± 0.19 in the AdCMV group ($p = 0.04$) (Fig. 4 J).

AdVammin increased the relative perfusion at rest in the treatment areas six days after the gene transfer

The relative perfusion at rest in the anterior wall six days after the gene transfer was 1.05 ± 0.08 in the AdCMV 1×10^{10} vp group and 1.24 ± 0.10 in the AdVammin 1×10^{10} vp group (Fig. 5). The difference was statistically significant ($p = 0.02$). During the adenosine-induced hyperemia, the relative perfusions in the anterior wall were 1.05 ± 0.15 and 1.07 ± 0.06 for AdCMV 1×10^{10} vp and AdVammin $1e10^{10}$ vp groups, respectively (Fig. 5). The

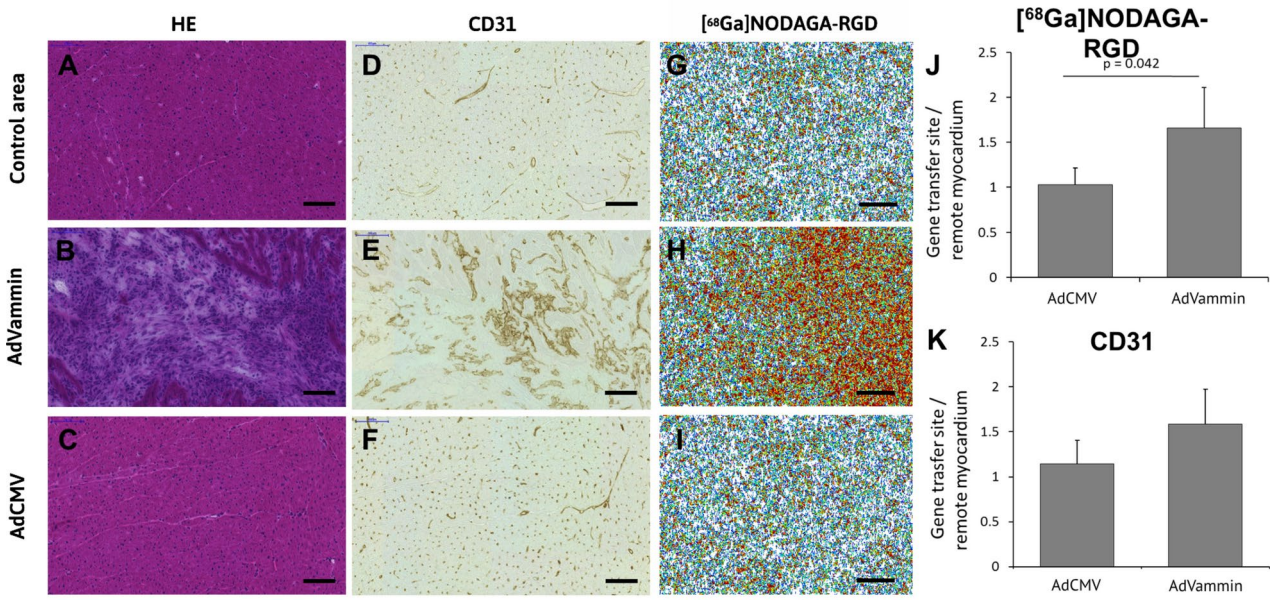

Fig. 4. Angiogenesis and myocardial [^{68} Ga]NODAGA-RGD-uptake in healthy porcine hearts**.** Compared with the remote myocardium (A and D) and the gene transfer site in the control group (AdCMV 1×10^{10} vp, C and F), there is a marked angiogenic response at the AdVammin gene transfer site (AdVammin 1×10^{10} vp, B and E). Blood vessel endothelium was detected by immunohistochemical staining with CD31 (D, E, F and K). Autoradiography shows increased [^{68} Ga]NODAGA-RGD uptake at the site of AdVammin gene transfer (H) as compared with the site of control gene transfer or the remote myocardium (G and I). The ratio of [^{68} Ga] NODAGA-RGD uptake in the gene transfer sites and the remote areas is significantly higher after AdVammin transfer than in the controls (J). Scale bars 100 µm (A-F) and 1000 µm (G-I). H&E = Hematoxylin and eosin.

difference was not statistically significant (p = 0.84). Representative pictures of the perfusion maps are presented in Fig. 5.

The absolute myocardial perfusion at rest in the anterior wall was 1.23 ± 0.38 mL/g/min in the AdCMV 1×10^{10} vp group and 1.5 ± 0.26 mL/g/min in the AdVammin 1×10^{10} vp group (Fig. 5). During adenosine-induced stress, absolute perfusions in the anterior wall were 1.67 ± 0.54 mL/g/min in the AdCMV 1×10^{10} vp group and 1.99 ± 0.28 mL/g/min in the AdVammin 1×10^{10} vp group (Fig. 5). However, the differences in the absolute myocardial perfusions were not statistically significant. In the posterior wall, the absolute perfusions at rest were 1.15 ± 0.29 mL/g/min and 1.21 ± 0.19 mL/g/min for the AdCMV 1×10^{10} vp and AdVammin 1×10^{10} vp groups, respectively. During adenosine-induced stress, absolute perfusions in the posterior wall were 1.57 ± 0.39 mL/g/min and 1.87 ± 0.27 mL/g/min for the respective groups. These differences were not statistically significant. A representative image of [^{68} Ga]NODAGA-RGD peptide uptake and myocardial perfusion at rest for the AdVammin group is shown in Fig. 6.

AdVammin increased ejection fraction in the ischemic hearts and had no effects on cardiac output

The mean EFs during dobutamine-induced stress six days after the gene transfer in the AdVammin and AdCMV groups were 48 ± 6.27% and 51.25 ± 4.11% (p = 0.42, Fig. 70. A). The baseline values before the gene transfer were 46.0 ± 5.35% and 43.5 ± 4.80%, respectively (Fig. 7A). However, the mean changes in EFs from baseline to six days were 2.25 ± 4.11% and 7.75 ± 1.5% (p = 0.045) in the AdCMV and AdVammin groups, respectively (Fig. 7B). At the same time point, the mean COs were 6.3 ± 0.97 L/min and 4.79 ± 0.13 L/min for AdCMV and AdVammin groups, respectively (Fig. 7C). Before the gene transfer, the baseline values for CO were 5.72 ± 0.96 L/min and 6.29 ± 0.44 L/min, respectively (Fig. 7C). Unlike in the EF, there was no statistically significant increase in the CO following the gene transfer. The mean changes in CO during stress were 1.91 ± 1.09 L/min and 1.33 ± 1.50 L/min (p = 0.55) for AdCMV and AdVammin groups, respectively (Fig. 7D).

The mean infarct sizes measured from photographs taken from cross-sections of the excised hearts incubated in tetrazolium chloride were 22.23 ± 8.45% and 19.86 ± 8.19% for AdCMV and AdVammin groups, respectively. Representative images of tetrazolium chloride incubated cross-sectioned hearts are shown in Figs. 7 E and F. The difference between the groups was not statistically significant (p = 0.66) (Fig. 7. I). The occluded bottleneck stent and anterior wall dyskinesia are shown in Figs. 7 G and H, respectively.

Discussion

The current study is the first to show that VEGFR-2 and NRP selective gene transfer with AdVammin induces potent angiogenesis in the myocardium in a clinically relevant large animal model. Also, this is the first study,

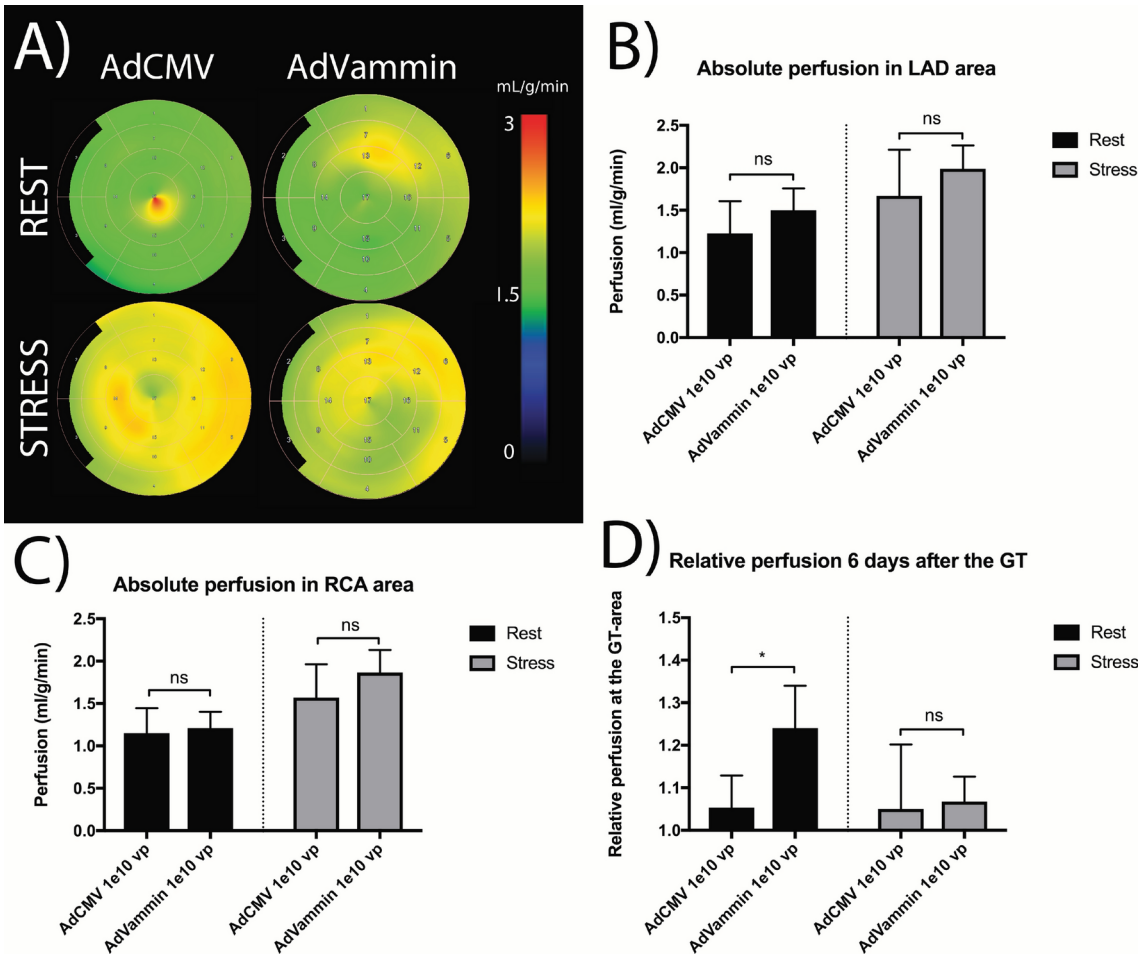

Fig. 5. AdVammin increased the relative perfusion at rest in the treatment areas six days after the gene transfer in healthy porcine hearts. The representative polar maps from the radio water-PET perfusion measurement are shown in A. The mean perfusion in the LAD and RCA areas corresponds to the treatment and control areas six days after the gene transfer for groups AdCMV 1×10^{10} vp and AdVammin 1×10^{10} vp are shown in B and C, respectively. The perfusion in the LAD area relative to the RCA area in rest was significantly increased in the AdVammin 1×10^{10} vp group compared to the AdCMV 1×10^{10} vp group (D). Statistical significance is marked by "ns" or asterisks (ns = $p > 0.05$ and * = $p \leq 0.05$).

to the authors' knowledge, to demonstrate fluoroscopically detectable angiogenesis in response to gene therapy in healthy myocardium.

In the first part of the study, the optimal AdVammin dosing was determined from the 2×10^{9} vp, 1×10^{10} vp, and 2×10^{10} vp. AdCMV 2×10^{10} vp was used as a control. The proangiogenic efficacy of the therapy was evaluated by measuring the mean capillary area from the CD31-stained IHC sections. Possible adverse effects were evaluated by cardiac echocardiography, modified miles assay, and by interpreting tissue morphology from the H&E-stained myocardial and safety sample sections. Surprisingly, AdVammin caused robust angiogenesis at very low viral doses compared to earlier studies that have used adenoviral VEGF-A, VEGF-B, or VEGF-D. In these studies, viral titers up to 2×10^{12}vp were used[9,28], whereas in the current study, even 2×10^{9} vp was sufficient to induce angiogenesis. Angiogenic effects were dose-dependent between the AdVammin doses 2×10^{9} vp and 1×10^{10} vp, but there was no significant difference between the doses 1×10^{10} vp and 2×10^{10} vp. However, there was a dose-dependence in the increased tissue permeability seen as pericardial fluid effusion and increases in relative permeability measured by modified miles assay. Thus, the AdVammin dose of 1×10^{10} vp was determined to be optimal for further studies as it resulted in robust angiogenesis with the least side effects.

VEGFR-2 and NRP selective AdVammin gene transfer with the dose of 1×10^{10}vp increased relative myocardial perfusion at the target area. However, the detected changes in myocardial perfusion were only modest compared to angiogenic effects as assessed by histological methods and fluoroscopy. Interestingly, despite their less potent angiogenic potential, AdVEGF-D and VEGF-B have been shown to be more potent in increasing myocardial perfusion in healthy myocardium[9,13]. This could result from some enlarged vessels induced by AdVammin being non-functional and thus not adequately perfused. The stealing effect may also explain the results. In this case, perfusion is inappropriately distributed as the blood flows according to a path of least resistance.

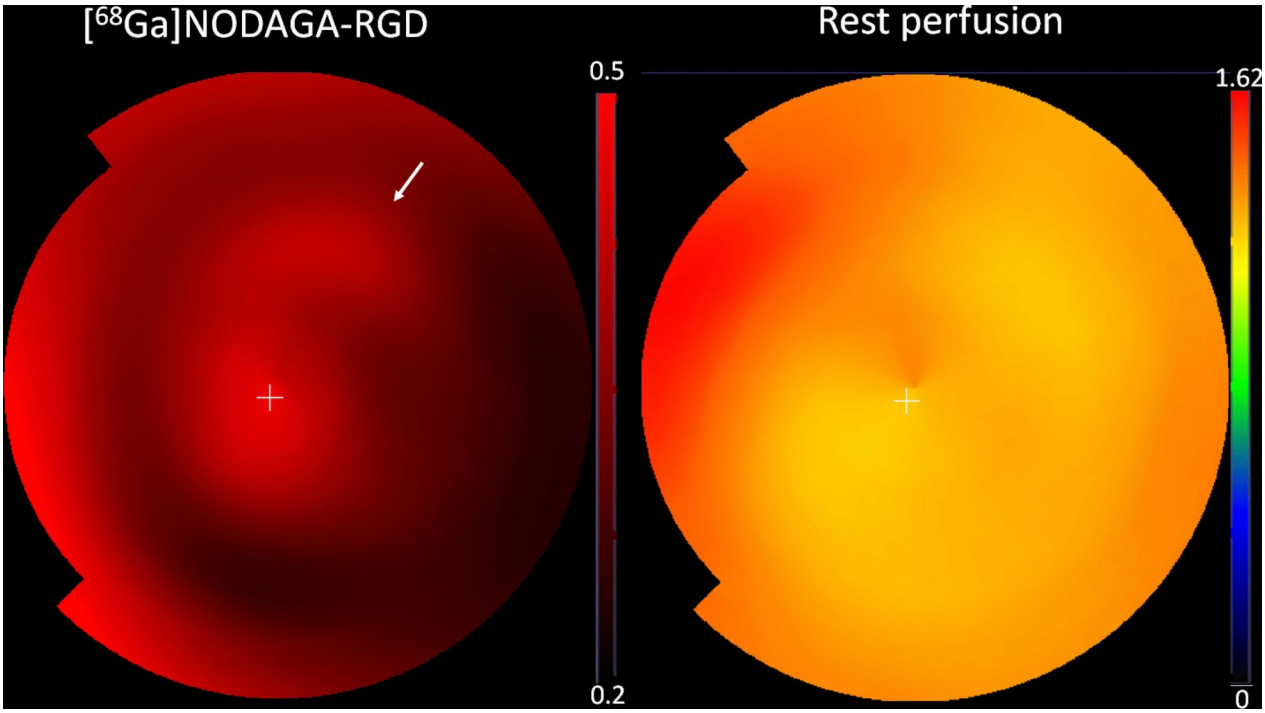

Fig. 6. Representative image of [^{68} Ga]NODAGA-RGD peptide uptake and myocardial perfusion at rest. Figure shows polar maps of myocardial [^{68} Ga]NODAGA-RGD uptake 40–60 min after injection (standardized uptake value, SUV) and myocardial perfusion at rest (mL/min/g). Anterolateral wall (injection site) is marked with an arrow.

The current study demonstrated that PET-imaging using [^{68} Ga]NODAGA-RGD peptide could detect enhanced angiogenesis in a clinically relevant large animal model. This finding could help to evaluate patient response to angiogenic therapies minimally invasively in clinical trials.

Gene transfer using AdVammin increased tissue permeability in a dose-dependent manner, with the largest doses resulting in the accumulation of pericardial fluid. In GO analysis of endothelial cells, AdVammin was shown to regulate genes that have previously been associated with vascular permeability, such as NR4A1, ESM1, BMPER, ANGPTL4 and PDE2A[29,30,31,32,33]. In future studies, the optimal low dose of AdVammin should be used to ensure proper angiogenic effects without harmful tissue edema and pericardial fluid accumulation. In addition, other approaches, such as pharmacological therapy, could be investigated to limit pericardial fluid accumulation without impairing the angiogenic effects.

Also, gene therapeutical methods may limit Vammin's adverse effects. Previous studies show that AdSlit2 gene transfers simultaneously with AdVammin reduce AdVammin's VEGFR-2-mediated capillary enlargement and limit the increase in vascular permeability in the rabbit skeletal muscle. Furthermore, AdSlit2 combined with AdVammin increased capillary sprouting, whereas AdVammin alone resulted in large capillaries with minimal sprouting. Overall, AdSlit2 combined with AdVammin results in more physiological angiogenic effects. Slit family and their Roundabout (Robo) receptors are regulators of vascular remodeling[22]. Also, EphB4 signal activation has given promising results for normalizing dysfunctional vascular growth when using high doses of VEGFs[34].

Angiogenesis involves endothelial cells, surrounding pericytes, and smooth muscle cells[35]. Recruitment of pericytes enables stabilization and maturation of the newly formed vessels[36]. For these reasons, pericyte coverage is considered an essential marker for vessel maturation[35]. In the AdVammin group, the enlarged vessels at the gene transfer areas had well-defined and prominent pericyte coverage compared to the control areas, as seen in the cd31-SMA-immunostaining. We also observed similar vessel enlargement with pericyte coverage after gene transfer to hindlimbs[21,22]. However, despite this prominent pericyte coverage, we observed evident tissue edema and vascular leakage at the gene transfer sites, as seen previously with other AdVEGF-therapies[9,22,28,37]. Also, despite the capillaries having extensive pericyte coverage, vessels seemed non-functional and not adequately perfused, as observed in the radiowater PET-imaging. Considering these results, pericyte coverage might not be an optimal indicator for vessel functionality or maturation in angiogenic therapies.

AdVammin increased the EF in stress six days after the gene transfer in the ischemic pigs. However, this was not accompanied by a similar increase in CO. This could be at least partially explained by the increased tissue edema in the AdVammin group, as the accumulated pericardial fluid might result in a cardiac-tamponade-like condition, where the volumes of the left ventricle are decreased thus resulting in increases of the EF.

In conclusion, this preliminary study demonstrated that AdVammin producing a VEGFR-2 and NRP-specific ligand is a potent inducer of angiogenesis in the porcine myocardium. These effects are achieved with

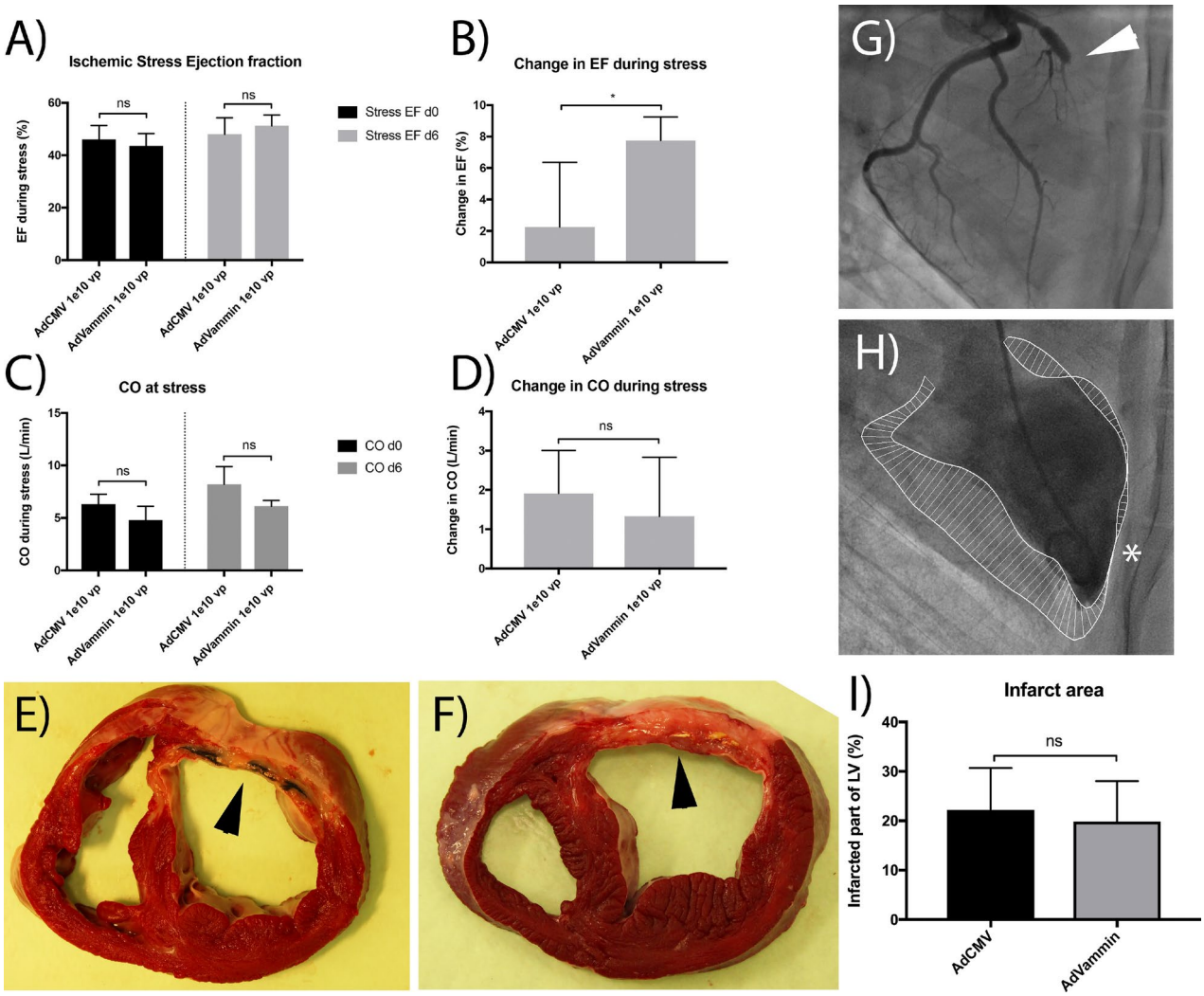

Fig. 7. Effects of AdVammin gene transfer on left ventricle function and infarct size in a porcine chronic ischemia model. A stent obstructing blood flow was placed in the LAD (white arrowhead, G). The ejection fraction (EF) and cardiac output (CO) were assessed using a left ventricular cine angiography (H). There were no significant differences in EF or CO between AdCMV 1×10^{10} vp and AdVammin 1×10^{10} vp groups six days after the gene transfers (A and C). However, the change in EF six days after the treatment was significantly higher in the AdVammin 1×10^{10} vp group when compared to the AdCMV 1×10^{10} vp group (B). In CO, no differences were observed (D). The infarct areas were calculated from tetrazolium chloride incubated cross-sections of the excised hearts. Representative pictures for AdCMV 1×10^{10} vp and AdVammin 1×10^{10} groups are shown in E–F. There was no significant difference in the infarct size between the groups 28 days after the gene transfer (I). T. The dyskinesia in the anterior wall is seen in the left ventricular cine angiography (white asterisk, H).

relatively low viral titers, making VEGFR-2 and NRP-specific ligands attractive candidates to induce therapeutic angiogenesis. However, the potential adverse effects of these treatments must be adequately controlled. Notably, this preliminary study had a limited sample size, limiting statistical power and thus making the study more exploratory in nature. Overall, further studies are needed to optimize the therapeutic effects of Vammin treatment.

Data availability

The datasets analyzed during the current study are available from the corresponding author upon reasonable request. The GO analysis datasets during the current study are available in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68535).

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Author contributions

PH, JN and AK performed the animal work. NJ and JP performed the laboratory work. NJ, PH, and JN analyzed angiography data, ultrasound images, capillary data, Modified Miles Assay data, infarct areas and radiowater PET images. MT, MG, AS and JK assisted with the radiowater PET-image analyses and analyzed [^{68} Ga] NODAGA-RGD PET images. JL performed the GO analysis and revised the paper. PT and TN assisted with the laboratory work, helped with the study design, and provided valuable information regarding their previous AdVammin studies. TR assisted with the animal work. NJ and PH did statistical analyses, wrote the manuscript, and designed the figures. SYH designed the study and revised the paper.

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Declarations

Competing interests

NJ is a shareholder of Saparo Translational Research Oy, a CRO for large animal studies.

Ethical statement

The Finnish National Animal Experiment Board and the Animal Experiment Board of the University of Finland approved all animal experiments. All animal experiments were in compliance with the ARRIVE guidelines. People involved in animal work have Felasa B or C certification.

Additional information

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