Capillary enlargement, not sprouting angiogenesis, determines beneficial therapeutic effects and side effects of angiogenic gene therapy

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Aims
Currently, it is still unclear which mechanisms drive metabolic benefits after angiogenic gene therapy. The side-effect profile of efficient angiogenic gene therapy is also currently incompletely understood. In this study, the effects of increasing doses of adenoviral (Ad) vascular endothelial growth factor-A (VEGF-A) were evaluated on vascular growth, metabolic benefits, and systemic side effects.

Methods and results
Adenoviral vascular endothelial growth factor-A or AdLacZ control was injected intramuscularly ($10^9$–$10^{11}$ vp/mL) or intra-arterially ($5 \times 10^{11}$ vp/mL) into rabbit ($n = 102$) hindlimb muscles and examined 6 or 14 days later. Blood flow, tissue oedema, metabolic benefits, and the structure of angiogenic vessels were assessed using ultrasound imaging, modified Miles assay, arterial blood gas and metabolite analyses, and light and confocal microscopy, respectively. Safety analyses included cardiac ultrasound, electrocardiograms, and blood and tissue samples. Sprouting angiogenesis was already induced with low AdVEGF-A concentrations, whereas higher concentrations were needed to reach efficient capillary enlargement and increases in target muscle perfusion. Interestingly, metabolic benefits, such as improved aerobic energy metabolism and decreased metabolic acidosis during exercise, after AdVEGF-A administration were highly correlated to the level of capillary enlargement but not to sprouting angiogenesis. Several systemic dose-dependent side effects, including transient increases in liver, kidney, and pancreatic enzymes, and signs of cardiac effects were observed.

Conclusion
Efficient capillary enlargement leading to significant increases in tissue perfusion is needed to gain metabolic benefits after angiogenic gene therapy. However, the risk of systemic side effects can increase as the efficiency of angiogenic gene therapy is improved. Importantly, the unstable wall structure of the newly formed vessels seems not to compromise the metabolic benefits.

Keywords
Angiogenesis • Vascular endothelial growth factor • Gene therapy • Side effects

Introduction
Ever since the discovery of factors inducing vascular growth, researchers have been enchanted by the idea of growing new blood vessels to replace blocked ones. It has now been clearly shown that angiogenic gene therapy can both induce the growth of functional vessels and increase skeletal muscle perfusion.1,2 However, it has not yet been fully accepted that angiogenic gene therapy with a single growth factor can induce functional vessels and metabolic benefits.3,4 Also, it has been unclear whether there are certain mechanisms, such as types of vascular growth or the stability of the formed vascular structures that especially drive metabolic benefits after angiogenic gene therapy.
Additionally, very little safety data are available from angiogenesis trials having used highly efficient vector constructs and shown clear angiogenesis in treated tissues. Detailed understanding of the side effects caused by efficient angiogenic gene therapy would be essential as manifold increased perfusion and vascular permeability in the treated tissues might, for example, enhance the release of the therapeutic protein from the target tissues. Structural reorganization of the vascular network, increased perfusion, and changes in local blood pressure gradients related to efficient angiogenesis might also cause haemodynamic changes that could reflect to the whole body.

In this dose escalation study, we have evaluated the effects of adenovirus expressing vascular endothelial growth factor-A (AdVEGF-A) on vascular growth, metabolic benefits, and systemic side effects. Members of the VEGF family are major stimulators of vascular growth and are among the most potential growth factors used currently and to be used in future clinical trials.

Methods
A more detailed description of the Methods section is provided in the Supplementary material online.

Gene transfers
Intramuscular (i.m.) gene transfers (GT) of adenoviruses encoding human VEGF-A or a β-galactosidase marker gene (LacZ) were performed to the semimembranosus and gastrocnemius muscles of New Zealand White rabbits (n = 102) during medetomidin (Domitor, 0.3 mg/kg, Orion) and ketamine (Ketalar, 20 mg/kg, Pfizer) anaesthesia. Increasing doses of 10^9–10^11 vp/mL with AdVEGF-A and 10^11 vp/mL with AdLacZ were used. In intra-arterial (i.a.) GT, 1.0 mL of 5 × 10^11 vp/mL AdLacZ or AdVEGF-A was injected selectively into the profound femoral artery. All animal experiments were approved by the Experimental Animal Committee of the University of Kuopio.

Contrast-enhanced ultrasound imaging of perfusion
Perfusion in the transduced and contralateral intact thigh and calf muscles was measured with Acuson Sequoia 512 and 15L8 transducer (Siemens) using a novel Cadence contrast pulse sequence application as described previously. Perfusion ratios between peak intensities in transduced and contralateral muscles were reported.

Blood sample analysis
Arterial blood samples were collected from a subgroup of animals before, and 6 and 14 days after GT. Clinical chemistry analysis was performed.}

Figure 1 Physiological vessel growth is induced with small adenoviral vascular endothelial growth factor-A (AdVEGF-A) doses, whereas large tortuous haemangioma-like lacunae and complex vascular networks are formed with the highest tested doses. Longitudinal confocal images of rhodamine-labelled Ricinus communis lectin perfused vascular structures (left column) and transverse light microscope images of CD31 immunostained vascular structures (right column) induced by different AdVEGF-A doses. (A) AdLacZ displays capillaries at normal size. (B) Sprouting angiogenesis (arrowheads) and some capillary enlargement (arrow) were seen with 1 × 10^9 vp/mL AdVEGF-A. (C) Sprouting (arrowheads) and stronger capillary enlargement (arrows) were seen in 5 × 10^9 vp/mL AdVEGF-A-transduced muscles. (D) Vast capillary enlargement (arrows) but still uniform vascular structures were present after 1 × 10^10 vp/mL AdVEGF-A. (E) Joining of enlarged capillaries to form blood lacunae (red arrows) was visible with 5 × 10^10 vp/mL AdVEGF-A. (F) Complex vascular structures and blood lacunae were abundant in 1 × 10^11 vp/mL AdVEGF-A-transduced muscles (red arrows). Scale bars 50 μm.
performed in the Eastern-Finland laboratory centre. For Astrup analysis, 1 ml of arterial blood was collected before and immediately after electrically stimulated exercise before, and 6 and 14 days after GT. The samples were immediately analysed using an ABL625 analyzer (Radiometer Copenhagen).

**Electrocardiography**

Electrocardiograms were registered using a 12-lead electrocardiograph (Sicard 460, Siemens) before, and 6 and 14 days after GT. Changes of each animal with respect to Day 0 were used to analyse group averages to reduce variance between animals.

**Modified Miles assay for evaluation of tissue oedema**

A modified Miles assay was used for the evaluation of tissue oedema at sacrifice as described previously. The results are expressed as ratios between the transduced and contralateral intact muscles using absorbances normalized to the weight of the muscle sample.

**Immunohistochemistry**

Monoclonal antibodies for VEGF-A (1:500, Santa Cruz), CD31 (1:50, Dako), β-galactosidase (1:250, Promega), and rabbit macrophages (RAM11, Dako, 1:200) with a trypsin pre-treatment were used in immunostainings on 7 μm-thick paraffin sections. Intra-arterially injected rhodamine-labelled Ricinus communis lectin was used for confocal microscopy on 50 μm-thick frozen sections.

Images were captured using either an Olympus AX70 microscope (Olympus Optical) or an Olympus IX81 confocal microscope and further processed for publication.

**In situ hybridization**

The localization of VEGF-A mRNA was shown by non-radioactive *in situ* hybridization (Roche Diagnostics Co.) from paraffin sections using a digoxigenin-labelled antisense and sense probes for VEGF-A according to the manufacturer’s instructions.

**Blood vessel measurements**

Capillary area (μm²) was measured from CD31 immunostained sections of semimembranosus muscles at ×200 magnification. The total area of arteries and veins of the muscle area (%) was quantified from α-SMA-stained sections of semimembranosus muscles at ×40 magnification covering the entire muscle.

**Measurement of RAM11-positive inflammatory cells**

The number of inflammatory cells was quantified from RAM11-immunostained histological sections at ×100 magnification using analySIS software (Soft Imaging System).

**Human vascular endothelial growth factor-A enzyme-linked immunosorbent assay**

Serum samples were analysed with hVEGF-A ELISA (R&D Systems) and further normalized to the amount of total protein in each sample according to the manufacturers’ instructions. The amount of total protein in each sample was quantified with BCA protein assay kit (Pierce).

**Statistical analyses**

Results are expressed as mean ± SEM. Statistical significance was evaluated using SPSS software (version 14.0) with a Kruskal–Wallis test.
followed by Mann–Whitney $U$ test where appropriate. Two-sided significances are expressed against the same time point AdLacZ unless otherwise indicated. Correlation analysis was performed using Spearman’s $\rho$ correlation test. A value of $P < 0.05$ was considered statistically significant.

**Results**

Capillary sprouting is induced with small adenoviral vascular endothelial growth factor-A doses whereas capillary enlargement requires higher local adenoviral vascular endothelial growth factor-A concentrations

AdLacZ-transduced muscles showed uniform capillary tubes arising from small arterioles with occasional branches, whereas AdVEGF-A-transduced muscles displayed a wide variety of angiogenic effects in rabbit hindlimbs 6 days after GT (Figure 1).

Increased capillary tube branching (i.e. sprouting angiogenesis; Figure 1, arrowheads) was detected with $1 \times 10^9$ vp/mL AdVEGF-A. Capillary enlargement (Figure 1, arrows) was clearly detectable with $5 \times 10^9$ vp/mL AdVEGF-A, but the arteriole–capillary hierarchy was still visible. $1 \times 10^{10}$ vp/mL and higher AdVEGF-A doses induced strong capillary enlargement and the hierarchy of end-arterioles and capillaries was lost. Aberrant, tortuous haemangioma-like blood vessel lacunae (Figure 1, red arrows) were even formed with the highest AdVEGF-A doses tested.

Different forms of angiogenesis varied also within the transduced muscles depending on the local VEGF-A concentration. Vascular endothelial growth factor-A immunostainings demonstrated that protein expression was not uniform in all parts of the transduced muscles (Figure 2). In AdLacZ-transduced control muscles, very little VEGF-A protein could be detected (Figure 2A). Instead, in AdVEGF-A-transduced muscles, strong signal showed that most of the produced protein was localized at the muscle borders, near the fascias (Figure 2B). The changes in capillary area seemed to correlate with the local VEGF-A concentration.

![Figure 3](https://example.com/figure3.png) Efficient capillary enlargement and increases in tissue perfusion lead to reduction in lactate formation and metabolic acidosis during exercise. (A) The level of sprouting angiogenesis was the same with all tested AdVEGF-A doses. (B) Capillary enlargement was dose-dependent after AdVEGF-A. (C) Increase in total vascular area was dose-dependent but reached a plateau at $5 \times 10^{10}$ vp/mL AdVEGF-A. (D) Increase in local tissue perfusion was dose-dependent after AdVEGF-A but plateaued already with $1 \times 10^{10}$ vp/mL AdVEGF-A. (E) Lactate formation and (F) metabolic acidosis during exercise were reduced dose-dependently after AdVEGF-A but were only significant with the $1 \times 10^9$ vp/mL dose. The maximal effects of AdVEGF-A were seen 6 days after GT and by 14 days most of the effects had returned to baseline. However, with total vascular area and perfusion, there were still significant increases at 14 days. Also, there seemed to be a reduction in acidosis still 14 days after GT. L-Z = AdLacZ, doses are reported for AdVEGF-A (vp/mL). $^*P < 0.05$ and $^{**}P < 0.01$ against same time point AdLacZ unless otherwise indicated.
Whereas a more uniform, dose-dependent vessel growth was induced in the middle of the muscle (Figure 2B, arrowheads), large blood vessel lacunae were formed near the VEGF-A-positive muscle fascias (Figure 2B, arrows).

The uneven protein distribution could not be explained by the cell population producing the VEGF-A protein as analysed by in situ hybridization which revealed that VEGF-A mRNA was produced by several cell types across the transduced muscle (see Figure 4).

Figure 4 High doses of AdVEGF-A have both local and systemic side effects. (A) Transient tissue oedema was highly dose-dependent after AdVEGF-A peaking at 6 days and resolving almost completely by 14 days. (B) Local tissue inflammation was dose-dependent and related to both the viral amount and VEGF-A concentration. Unlike most of the GT-induced side effects, the inflammatory response was not alleviated by 14 days. (C–E) Transient increases were observed in liver (ALT), kidney (CREA), and pancreatic enzymes (AMYL) with AdVEGF-A at 6 days. (F–H) Transient reductions were observed in arterial blood haemoglobin, pH, and Na levels were also observed. (I and J) Additionally, significant hyperkalaemia (K) and hypocalcaemia (Ca) were detected at 6 days. ALT, alanine transaminase; CREA, creatinine; AMYL, amylase; Na, sodium; K, kalium; Ca, calcium. L-Z, AdLacZ, doses are reported for AdVEGF-A. *P < 0.05 and **P < 0.01 against same time point AdLacZ unless otherwise indicated.
Supplementary material online, Figure S1). It was also tested whether a more uniform distribution of AdVEGF-A within the target muscles could be achieved by an i.a. GT, but the efficacy of this gene delivery method was poor and no significant angiogenic effects could be detected in AdVEGF-A-transduced muscles when compared with AdLacZ controls (see Supplementary material online, Figure S2).

**Metabolic benefits of angiogenic gene therapy are dependent on capillary enlargement and efficient increases in tissue perfusion**

When quantified, all tested AdVEGF-A doses induced equal capillary sprouting 6 days after GT (Figure 3A). Instead, the effects on maximal capillary enlargement were highly dose-dependent up to the highest tested AdVEGF-A dose (Figure 3B). Total vascular area, including capillaries, arteries, and veins, as well as increases in perfusion had a dose-dependent increase after the first tested doses but then reached a plateau after $5 \times 10^{10}$ and $1 \times 10^{10}$ vp/mL AdVEGF-A (Figure 3C and D), respectively. The increases in perfusion were best correlated with the increase in capillary area, then with the changes in total vascular area and less with the level of sprouting angiogenesis (Spearman’s $\rho$ correlations 0.786**, 0.718**, and 0.573**, respectively, $**P < 0.01$). Importantly, lactate formation and metabolic acidosis during exercise were decreased dose-dependently 6 days after AdVEGF-A GT (Figure 3E and F). Unlike with all other quantified parameters where all tested AdVEGF-A doses had significant differences in comparison to the AdLacZ controls, only the highest tested AdVEGF-A dose ($1 \times 10^{11}$ vp/mL) had a significant effect on lactate formation and metabolic acidosis over the AdLacZ controls. The changes in metabolic parameters correlated best with the changes in capillary area and then with total vascular area and perfusion but not with capillary sprouting (Spearman’s $\rho$ correlations for lactate formation 0.9142**, 0.8888**, 0.841**, and 0.3803, respectively, $**P < 0.05$ and $**P < 0.01$).

Two weeks after the GT, most of the described angiogenic changes had already returned to baseline due to the transient VEGF-A expression by adenovirus. However, significantly increased total vascular area and perfusion could still be observed (Figure 3C and D). Also, a decrease in exercise-induced metabolic acidosis was observed with AdVEGF-A $10^9$ vp/mL (Figure 3F).

**Side effects after angiogenic gene therapy are highly dose-dependent and mediated via both blood and lymphatic distribution of the gene construct**

Adenoviral vascular endothelial growth factor-A GT had both local and systemic side effects. Dose-dependent tissue oedema in the transduced limbs was found 6 days after AdVEGF-A GT (Figure 4A). Tissue oedema formation correlated best with the increase in capillary area, but less with the increases in perfusion or total vasculature, and not with capillary density (Spearman’s $\rho$ correlations 0.824**, 0.679**, 0.558*, and 0.297, respectively, $**P < 0.05$ and $*P < 0.01$). The accumulation of RAM11 + inflammatory cells in the transduced muscles was observed 6 and 14 days after both AdVEGF-A and AdLacZ GT (Figure 4B). Transient increases in liver (Figure 4C), kidney (Figure 4D), and pancreatic (Figure 4E) enzymes were observed 6 days after AdVEGF-A $1 \times 10^{11}$vp/mL GT. A dose-dependent decrease in systemic blood haemoglobin that returned to baseline by 14 days was also observed after AdVEGF-A (Figure 4F). The highest tested dose of AdVEGF-A also significantly reduced systemic blood pH (Figure 4G) and induced transient electrolyte changes such as hyponatraemia (Figure 4H), hyperkalaemia (Figure 4I), and hypocalcaemia (Figure 4J) at 6 days.

Some of the highest dose AdVEGF-A ($10^{11}$ vp/mL) animals also died during the 14-day follow-up. The deaths occurred around the 6-day maximal effect and the deaths seemed to be related to the animal weight, so that with the smallest animals (weight 2–2.5 kg), the death rate was 60% and with the medium-sized

![Figure 5](image_url) Intramuscular AdVEGF-A GT to the hindlimb has cardiac effects. (A) Ultrasound analysis of ejection fractions (EF) of animals with GT in their hindlimbs showed significantly reduced EF after AdVEGF-A when compared with AdLacZ controls. The greatest EF reduction was detected at 6 days but some was still visible at 14 days. (B) A tendency towards left ventricular wall thickening (LVDT) was also observed in the AdVEGF-A animals. (C) Electrocardiograms showed an increase in left ventricular mass in AdVEGF-A-transduced animals as analysed by the Sokolow–Lyon method ($S_{V1} + R_{V5-LV}$). $*P < 0.05$ and $**P < 0.01$ against same time point AdLacZ unless otherwise indicated.
animals (weight 2.5–3 kg) 33%. Among the biggest animals (3–3.5 kg), the death rate was zero, although, for example, significant electrolyte changes were registered. Thus, also the cardiac status of the animals was studied. Cardiac ultrasound revealed significant reductions in the ejection fraction of AdVEGF-A $1 \times 10^{11}$ vp/mL animals when compared with AdLacZ controls both 6 and 14 days after GT (Figure 5A). Also, a tendency towards an increase in left ventricle diastolic wall thickness was observed after AdVEGF-A $1 \times 10^{11}$ vp/mL GT with the ultrasound analysis (Figure 5B). The analysis of electrocardiograms of the animals also indicated left ventricular strain as shown by increased left ventricular voltages (Figure 5C).

Side effects were found to be mediated by the release of the gene construct via both blood and lymphatic vessels. Immunohistochemical analysis of lymph nodes 6 days after AdLacZ GT revealed transgene expression after an i.m. GT (Figure 6A and B). Dose-dependent, up to 83-fold, increases in systemic blood VEGF-A levels were also observed after AdVEGF-A when compared with the AdLacZ control (Figure 6C). However, in immunohistochemical analysis, increased VEGF-A expression and signs of angiogenesis were only found in the lymph nodes closest to the GT sites but not from distal lymph nodes or from the myocardium (Figure 6D–K).

Discussion

From previous studies, local VEGF-A concentration and blood flow are known to modulate the structure of angiogenic vessels. In this study, increasing the overall viral dose of AdVEGF-A with continuous flow displayed a change in the type of angiogenesis from sprouting angiogenesis to capillary enlargement and even to the formation of blood-filled lacunae. The level of sprouting angiogenesis was similar with all tested AdVEGF-A doses and correlated less with the increases in tissue perfusion and metabolic benefits than the changes in maximal capillary and total vascular area. The importance of vascular enlargement on improved tissue energy metabolism is explained by the principles of Poiseuille’s law which states that the rate of blood flow is proportional to the fourth power of the vessel radius. This implies that growth of the whole vascular tree and especially capillary enlargement, leading to efficient increases in local tissue perfusion, need to be stimulated in efficient angiogenic gene therapy (Figure 7).

Significant changes in vessel growth and perfusion could be achieved with all tested AdVEGF-A doses. However, reaching significant changes in the metabolic parameters, such as lactate formation or metabolic acidosis, was more complicated. This likely reflects that not only the blood supply to the muscle determines

![Figure 6](image-url) Side effects of angiogenic gene therapy are mediated by both blood and lymphatic release of the gene product. (A and B) Histological sections of popliteal lymph nodes of an AdLacZ-transduced animal (A) showing LacZ gene expression (arrows) and a control lymph node (B). (C) Quantitative analysis on blood VEGF-A levels revealed a dose-dependent increase in systemic VEGF-A concentration in AdVEGF-A-transduced animals. *p < 0.05. (D–G) VEGF-A expression in AdLacZ $1 \times 10^{11}$ vp/mL transduced muscle (D), and in the popliteal (E) and thoracic lymph nodes (F) and the myocardium (G) of the same animal showing only faint VEGF-A expression on arterial walls (arrow) and normal-sized capillaries (arrowheads). (H–K) VEGF-A expression in AdVEGF-A $1 \times 10^{11}$ vp/mL transduced muscle (H), and in the popliteal (I) and thoracic lymph nodes (J) and the myocardium (K) of the same animal showing increased VEGF-A expression (arrows) and enlarged capillaries (red arrowheads) in the transduced muscle and its adjacent lymph node, but normal expression and normal-sized capillaries (black arrowheads) more distal to the GT area. Scale bars 100 μm.
the energy metabolism. Additionally, a dose-dependent haemodilution, likely due to increased vascularity, was observed in the AdVEGF-A-transduced animals, which may reduce the amount of oxygen carried by a blood unit. This may counteract the positive effects of gene therapy for as long as erythropoiesis cannot compensate the reduced red blood cell concentration.

Whereas some heterogeneity of the angiogenic response in the transduced muscles could be observed, according to the local distribution of the growth factor as previously published, increasing the adenoviral (Ad) dose resulted also in a more uniform and evenly spread capillary enlargement. Localization of most of the growth factor to the muscle fascias in the transduced muscles was not explained by the distribution of the transduced cells producing the growth factor but rather the distribution of the secreted growth factor and the pressure gradients inside the muscle. The use of i.a. GT did not improve the distribution of the growth factor due to overall poor transduction efficacy. Thus, i.m. GT should be favoured in future trials as they are clearly more potent than i.a. GT. Also, some level of heterogeneity in the angiogenic response may have to be accepted in order to reach an efficient overall effect in the transduced muscles.

Interestingly, the increasing complexity of the vascular network with the highest VEGF-A doses did not impair target muscle energy metabolism as lactate formation and metabolic acidosis during exercise were dose-dependently smaller and only significant with the highest tested doses, although there was no more increase in regional tissue perfusion. Improved metabolism despite the apparent plateau in regional tissue perfusion may be explained by the fact that the higher AdVEGF-A doses induced more widespread angiogenic changes than lower concentrations, thus resulting in an improved metabolic net effect. This highlights the need for high growth factor concentrations in the transduced muscles and subsequently also implies the need for the use of highly efficient gene delivery vehicles. Importantly, the metabolic findings also suggest that the newly formed vessels are functional and capable of exchanging nutrients and metabolic products even if the wall structures are not yet fully stabilized and could not stand VEGF deprivation.

After the initial growth stimulus, the formed vascular network is pruned to suit the needs of the target tissue. In the skeletal muscle, the level of perfusion in resting conditions is very low and thus it was natural to see regression of excess capillaries by 14 days as the AdVEGF-A expression ceased. A longer but less prominent angiogenic stimulus might be induced using other vectors, such as AAV. However, according to our results, capillary sprouting alone is not sufficient to cause metabolic benefits. The initially strong growth stimulus in the capillaries activated the growth of the whole vascular tree and increased tissue perfusion, both parameters that were still significantly increased 14 days after GT. This preconditioning of the vascular tree has been shown to enhance recovery from later ischaemia.1

Previously, the main side-effect concerns after angiogenic gene therapy have been the possible long-term complications, such as activation of malignancies, due to transgene biodistribution.11 Here, we show that transgene release from the target tissue and side-effect prevalence are highly increased as more efficient angiogenesis is pursued. The release of VEGF-A from the target tissues is somewhat surprising as VEGF-A is a matrix bound growth factor that one would not expect to diffuse easily away from the transduced tissue.13 Previously, only very small amounts of VEGF-A have been found in circulation after i.m. GT.13,14 Increased perfusion and vascular permeability in the transduced tissues might have facilitated VEGF-A release from the muscles into the systemic circulation. Additionally, the virus itself was found to be taken up by the lymphatic system and transported to the lymph nodes creating a new transgene production platform.

Acute side effects that could be especially related to angiogenesis and rapid changes in haemodynamics were also observed. Tissue oedema in AdVEGF-A-transduced limbs was strongly amplified as capillary enlargement increased and it could also explain some of the observed electrolyte changes. Oedema can be avoided by titration of the overall viral dose but a subsequent loss of angiogenic and metabolic efficacy may then follow. Local inflammation in the transduced tissues was related to the Ad dose as also AdlacZ control muscles had inflammatory cell infiltration. This highlights the use of highly efficient growth factors in angiogenic gene therapy since then smaller viral doses can be used. High AdVEGF-A doses were also found to have several systemic side effects. Transient liver and kidney enzyme elevations have been reported previously to be related to the use of the Ad vector,15 but here these changes were related rather to the VEGF-A protein as AdlacZ controls displayed no such changes. Additionally, high AdVEGF-A doses were found to induce electrolyte changes and cardiac effects likely via modified haemodynamics and increased blood volume induced by the hindlimb angiogenesis. In support of this, no change in VEGF-A expression was observed...
in the distal lymph nodes or in the myocardium. Also, the peak for both electrolyte changes and reduction in ejection fraction took place 6 days after GT, when the angiogenesis response was the greatest. Fourteen days after GT, when most of the angiogenic changes had returned to baseline, the electrolyte changes had resolved and also the cardiac ejection function showed some improvement. However, signs of compensatory left ventricular thickening were observed in cardiac ultrasound and analysis of electrocardiograms at 14 days. On the basis of these results, no direct dose recommendations can be made to patients regarding the appearance of side effects, especially to those who might suffer from previous cardiac problems and might thus be more prone to develop cardiac-related side effects. Also, further studies are needed to investigate the exact pathological mechanisms behind the observed side effects. However, these data suggest that such side effects can occur and patients in future trials should be carefully monitored to detect any changes.

In summary, the optimization of the overall viral dose in angiogenic gene therapy leads to physiological vessel growth and reduction in side effects such as tissue oedema. However, reaching significant metabolic benefits requires efficient capillary enlargement and increases in tissue perfusion, and thus relatively high AdVEGF-A concentrations. Thus, this study demonstrates that when efficient gene constructs are used also metabolic benefits can be achieved but this will also warrant careful monitoring of side effects that will evidently increase as the efficiency of angiogenic gene therapy is improved.

Supplementary material
Supplementary material is available at European Heart Journal online.

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