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Kirsi Muona

Safety of VEGF Gene Therapy in Cardiovascular Diseases

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KIRSI MUONA

Safety of VEGF Gene Therapy in Cardiovascular Diseases

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ABSTRACT

VEGF gene therapy is a promising alternative treatment method for patients with severe cardiovascular diseases. Pro-angiogenic growth factors involve theoretical risks, such as acceleration of pathological angiogenesis in tumour growth and retinopathy, inflammatory responses and tissue oedema that need to be carefully assessed. VEGF-A is the most commonly used growth factor in clinical gene therapy trials and its short-term safety has been investigated in several studies. However, there is limited data available regarding long-term effects and safety of VEGF gene therapy.

In this work we investigated the safety and effects of VEGF gene transfer. Primary endpoints were to assess long-term safety of adenoviral (Ad) and plasmid/liposome (P/L) mediated VEGF-A gene therapy in the treatment of coronary artery disease (CAD) and peripheral artery disease (PAD) in patients not suitable for revascularization. In addition, we investigated the short-term safety and feasibility of the very first adenovirus mediated VEGF-D$^{\Delta N \Delta C}$ gene transfer performed by using transseptal puncture in no-option CAD patients. Secondary endpoint was to study long-term efficiency and effects of VEGF-A gene transfer in these patient groups.

The incidence of cancer, retinopathy or diabetes did not increase significantly in AdVEGF-A or P/LVEGF-A groups compared to the control groups in 8- and 10-year follow-ups (CAD and PAD, respectively). Furthermore, there was no significant difference in mortality between the groups. In CAD patients, no statistically significant difference in working ability, exercise tolerance, or major cardiac events was seen between the groups. In PAD patients no difference in the number of amputations, vascular interventions, or exercise tolerance was detected between the treatment and control groups.

In the short-term interim safety evaluation of VEGF-D$^{\Delta N \Delta C}$ gene transfer laboratory parameters, clinical examination and transthoracal echocardiography (TTE) as well as severe adverse events (SAE) were assessed. According to the results, transseptal puncture proved to be a feasible and well tolerated method to deliver gene into the left ventricular myocardium. No serious arrhythmias during or after the procedure were detected. Transient elevation in body temperature and inflammatory parameters were seen in both control and treatment groups. Minimal pericardial effusion was seen in a few patients in the treatment group. However, this was resolved spontaneously and required no further procedures.

In conclusion, our studies suggest that local VEGF-A gene transfer for CAD and PAD is safe in long-term follow-up and does not increase the incidence of cancer, diabetes, or its complications. No difference in mortality, major cardiovascular events, or exercise tolerance was seen in either of the studies when compared to the control groups. Furthermore, VEGF-D$^{\Delta N \Delta C}$ gene therapy by using transseptal puncture route is feasible and well tolerated.

National Library of Medical Classification: QU 560, WG 166
Medical Subject Headings: Vascular Endothelial Growth Factor A; Vascular Endothelial Growth Factor D; Gene Transfer Techniques; Genetic Therapy/adverse effects; Cardiovascular Diseases; Coronary Artery Disease/therapy; Peripheral Vascular Diseases/therapy; Follow-Up Studies; Neoplasms; Diabetic Retinopathy

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TIIVISTELMÄ

VEGF- geenihoito on lupaava uusi hoitomuoto vaikea-asteisia sydän- ja verisuonisairauksia sairastaville potilaille. Verisuonten kasvua edistäviin eli angiogeneettisiin kasvutekijöihin liittyy kuitenkin teoreettisia riskejä, kuten verisuonikasvun patologinen lisääntyminen pahanlaatuisissa kasvaimissa ja diabeettisessa silmänpohjasairaudessa/retinopatiassa. Lisäksi geenihoitoon liittyen on havaittu immunologisia reaktioita ja geenihoidolla hoidetun kohdekudosten turvotusta. VEGF-A on eniten käytetty verisuonten kasvutekijä kliinisissä tutkimuksissa ja sen turvallisuutta on tutkittu useissa lyhyen aikavälin seurantatutkimuksissa. Pitkäaikaisista vaikutuksista ja hoidon turvallisuudesta on kuitenkin vain vähän tutkimustietoa saatavilla.
Tässä työssä ensisijainen tavoite oli selvittää virus (adenovirus, Ad)- ja rasvapartikkeli (plasmidi/liposomi, P/L)-välitteisen VEGF-A geenihoidon turvallisuutta sepelvaltimo- ja ääreisvaltimotautia (ASO) sairastavilla potilailla, joille muut hoitomuodot ovat riittämättömiä tai soveltumattomia. Toisena tavoitteena oli tutkia VEGF-A geenihoidon tehokkuutta ja pitkäaikaisvaikutuksia näissä potilasryhmissä. Lisäksi tutkimme toisen tyyppisen verisuonikasvutekijän eli VEGF-D$^{\Delta N \Delta C}$ -geenihoidon turvallisuutta ja hoitomenetelmän käytettävyyttä vaikea-asteista sepelvaltimotautia sairastavilla potilailla. Kyseessä oli ensimmäinen kliininen tutkimus kyseisellä geenillä.
Kymmenen vuoden seurannassa ei havaittu merkitseviä eroja syövän, silmänpohjasairauden tai diabeteksen esiintyvyydessä geenihoidon saaneiden potilaiden ja kontrollihenkilöiden välillä. Kuolleisuudessa ei myöskään todettu tilastollista eroa ryhmien välillä sepelvaltimo- tai ASO-potilaiden kohdalla.
Hoidon tehokkuutta mittaavassa tutkimuksessa VEGF-A geenihoitoa saaneilla potilailla ei todettu tilastollisesti merkittävää eroa rasituksen siedon, sydäntapahtumien tai työkykyisyyden suhteen hoito- ja kontrolliryhmien välillä. Samoin ASO-potilailla ei todettu merkittäviä eroavaisuuksia amputaatioiden, vaskulaaritoimenpiteiden tai fyysisen suorituskyvyn suhteen.
VEGF-D$^{\Delta N \Delta C}$ tutkimukseen liittyvän välianalyysin tulokset osoittivat käytetyn geeninsiirtomenetelmän olevan käyttökelpoinen ja hyvin siedetty hoitomuoto vaikeassa sepelvaltimotaudissa. Vakavia rytmihäiriöitä ei todettu toimenpiteeseen liittyen. Ohimenevää lämmön ja tulehdusarvojen nousua todettiin yhtä paljon sekä geenihoitoa saaneilla että kontrollipotilailla. Lievää ja nopeasti ohimenevää sydänpussin nesteilyä todettiin yksittäisillä geenihoitoa saaneella potilaalla.
Tutkimuksemme osoittavat paikallisen VEGF-A geenihoidon olevan pitkällä aikavälillä turvallinen hoitomuoto sepelvaltimo- ja ASO-tautia sairastavilla potilailla. Lisäksi uusi sydänlihaksen sisäinen VEGF-D$^{\Delta N \Delta C}$ -geenihoito on käyttökelpoinen ja hyvin siedetty menetelmä vaikeaa sepelvaltimotautia sairastavilla potilailla, joille ei ole tarjolla muuta hoitovaihtoehtoa.

Yleinen Suomalainen Asiasanasto: geeniterapia, sydän- ja verisuonitaudit, turvallisuus, seurantatutkimus, syöpätaudit, diabetes

To my parents

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II Muona K, Mäkinen K, Hedman M, Manninen H, Ylä-Herttuala S. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. *Gene Ther 19:392-395, 2012.*

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Contents

1 INTRODUCTION 1
2 REVIEW OF THE LITERATURE 3
2.1. Normal development of blood vessels 3
2.1.1. Vasculogenesi 3
2.1.2. Angiogenesis 3
2.1.3. Arteriogenesis 4
2.2. Atherosclerosis 5
2.3. Coronary artery disease 7
2.3.1. General aspects 7
2.3.2. Treatment 8
Risk factor management 8
Pharmacological therapy 8
Exercise training 9
Revascularization 9
Neurostimulation 10
2.4. Peripheral artery disease 10
2.4.1. General aspects 10
2.4.2. Treatment 11
Risk factor management 11
Pharmacological therapy 12
Exercise training 12
Revascularization 12
Neurostimulation 12
2.5. Gene Therapy 13
2.5.1. Vectors 13
Viral vectors 13
Non-viral vectors 15
2.5.2. VEGF family 16
VEGF-A 16
VEGF-B 17
VEGF-C 17
VEGF-D 17
PlGF 18
2.5.3. Other growth factors 19
PDGF 19
FGF 19
HGF 19
IGF 20
2.6. Clinical VEGF trials 20
2.6.1. Coronary artery disease 20
2.6.2. Peripheral artery disease 22
2.7. Safety aspects and ethics of gene therapy 23
2.7.1. Ethical aspects 23
2.7.2. Safety aspects 24

Cancer 23
Arthritis 25
Diabetic retinopathy and macular oedema 26
Inflammation 26
Oedema 27
3 AIMS OF THE STUDY 29
4 METHODS 31
4.1. Vectors and gene transfer 31
4.2. Long-term follow-up of VEGF-A gene transfer 32
4.2.1. Patient selection 32
4.2.2. Data collection and questionnaire 32
4.3. Short-term follow-up of AdVEGF-$D^{\Delta N \Delta C}$ gene transfer 32
4.3.1. Patient selection and screening 32
4.3.2. Gene transfer 33
4.3.3. Safety protocol 33
4.4. Statistical analysis 34
5 RESULTS 35
5.1. Baseline characteristics and patient demographics 35
5.1.1. VEGF-A trials 35
5.1.2. Peripheral artery disease 35
5.1.3. AdVEGF-$D^{\Delta N \Delta C}$ trial 36
5.2. Efficacy and long-term effects 36
5.2.1. VEGF-A gene therapy for coronary artery disease 36
Working ability 36
Exercise tolerance 38
Major cardiovascular events 38
5.2.2. VEGF-A gene therapy for peripheral artery disease 39
Invasive procedures and amputation 39
Other effects 39
5.3. Safety 39
5.3.1. Long-term safety of VEGF-A gene therapy 39
5.3.2. Procedural and short-term safety of AdVEGF-$D^{\Delta N \Delta C}$ gene therapy 40
6 DISCUSSION 43
6.1. Long-term safety of VEGF-A gene therapy 43
6.2. Effects on exercise tolerance and major cardiovascular events 44
6.3. Short-term safety of AdVEGF-$D^{\Delta N \Delta C}$ gene therapy 45
6.4. Ethical aspects 47
7 SUMMARY 49
8 REFERENCES 51

Abbreviations

AAV	Adenoassociated virus
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
Adv	Adenovirus
ALD	Adrenoleukodystrophy
ALI	Acute limb ischemia
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANG	Angiotensin
ASA	Acetylsalicylic acid
BET	Bicycle exercise test
CABG	Coronary bypass graft
CAD	Coronary artery disease
CEA	Carcinoembryonic antigen
CCS	Canadian cardiovascular Society
CCU	Cardiac care unit
CLI	Critical limb ischemia
CMV	Cytomegalovirus
CRP	C-reactive protein
CTA	Computed tomopraphy angiogram
DLL4	Delta like ligand 4
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECG	Electrocardiography
ECM	Extracellular matrix
ESC	European Society of Cardiology
FDA	Food and Drug Administration (USA)
FGF	Fibroblast growth factor
GCP	Good clinical practice
GH	Growth hormone
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HSPG	Heparin sulphate proteoglycan
HSV	Herpes simplex virus
ICD-10	International classification of diseases-10
IGF	Insulin like growth factor
IL	Interleukin
IL2RG	Interleukin-2 receptor subunit gamma
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LV	Left ventricular
MACE	Major cardiovascular event
MEF2	Myocyte enhancer factor-2
MI	Myocardial infarction
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging

NK	Natural killer
NO	Nitric oxide
OTCD	Ornithine transcarbamylase deficiency
PAD	Peripheral artery disease
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
P/L	Plasmid/liposome
PlGF	Placental growth factor
PSA	Prostate specific antigen
PTA	Percutaneous transluminal angioplasty
RhVEGF	Recombinant vascular endothelial growth factor
RNA	Ribonucleic acid
SAE	Serious adverse event
SCID-X1	X-linked severe combined immunodeficiency
SCM	Smooth muscle cell
SCS	Spinal cord stimulation
SET	Set exercise treatment
$TcOP_2$	Transcutaneous oxygen pressure
TENS	Transcutaneous electrical nerve stimulation
TGF	Transforming growth factor
TIA	Transient ischemic attack
TNF	Tumour necrosis factor
TTE	Transthoracal echocardiography
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vpu	Viral particle unit

1 Introduction

Cardiovascular diseases are the leading cause of death and morbidity in developed countries. The incidence is also constantly increasing in developing countries. According to the World Health Organization's statistics, 17,3 million people died of cardiovascular diseases in 2008 worldwide and the number is estimated to reach 23,3 million by the year 2030 [WHO 2008].

Pharmacological therapy and revascularization offer an efficient treatment and improve the quality of life of patients with ischemic cardiovascular diseases. However, there are an increasing number of patients that do not benefit sufficiently from these treatment options and remain symptomatic despite maximal medication. Additionally, due to advanced disease and diffuse atherosclerotic plaques, or high risks for operations, revascularization may not be suitable [Andréll et al. 2011].

Gene therapy has been investigated as a novel treatment method for cardiovascular diseases. Vascular endothelial growth factor (VEGF) is a growth factor produced by endothelial cells to regulate angiogenesis and lymphangiogenesis. In adults, VEGF secretion is induced mostly by hypoxia. VEGF also induces pathological angiogenesis from pre-existing vessels. In embryo, VEGF regulates natural development of arteries, veins and lymphatic vessels. The members of VEGF family are the most selective activators of angiogenesis. VEGF-A has shown the fastest response to hypoxia and is therefore one of the most widely investigated growth factors in cardiovascular diseases. Furthermore, other growth factors such as Fibroblast growth factors (FGF) and Platelet derived growth factors (PDGF) have been studied as potential therapeutic agents [Zachary and Morgan 2011].

Gene therapy has shown promising results in preclinical studies. Substantial improvement in collateral vessel growth in animal models has been seen in multiple trials [Rissanen and Ylä-Herttuala 2007; Whitlock et al. 2004]. The results of clinical trials have been fairly modest, although some improvement, for instance, in myocardial perfusion has been detected [Hedman et al. 2003]. Replication deficient viral vectors have proven to be the most efficient in terms of gene transduction and expression [Giacca et al. 2012]. The risk of immunological responses is, however, higher compared to non-viral vectors. Non-viral vectors have a better safety profile and fewer adverse effects, but they have not reached the same efficiency in gene transfer as viral vectors [Wang et al. 2012].

In addition to immunological responses, there are theoretical risks associated with VEGF mediated gene therapy, such as tumour growth, induction of diabetic retinopathy, and oedema [Ylä-Herttuala et al. 2007]. Indeed, VEGF is prominently expressed in

malignant tumours and enables the rapid and uncontrollable vessel growth inside the tumour. VEGF suppressing gene therapy has improved the prognosis of various types of cancers [Gatson et al. 2012] and reduced the progression of retinopathy [Davis et al. 2009]. Clinical cardiovascular VEGF trials have not shown increase in the incidence of cancer in short-term follow-ups. Also short-term safety aspects of VEGF-A are well known and no major safety concerns have been reported [Stewart et al. 2006; Hedman et al. 2003; Mäkinen et al. 2002]. However, only limited data of the long-term safety effects are available.

The purpose of this work is to investigate the long-term safety aspects and efficiency of local VEGF-A gene therapy in the treatment of CAD and PAD patients. In addition, the short-term and procedural safety of novel VEGF-$D^{\Delta N \Delta C}$ therapy in no-option CAD patients is studied. A profound evaluation of safety is indispensable considering wider therapeutic use and contributes to the better understanding of long-term effects of gene therapy.

2 Literature review

2.1 NORMAL DEVELOPMENT OF ARTERIES

2.1.1 Vasculogenesis

Vasculogenesis is defined as the physiological formation of vascular structures during embryonic development concerning the development of arteries, veins, and lymphatic vessels [Carmeliet and Jain 2011]. All blood and endothelial cells arise from embryonic mesenchymal tissue known as angioblasts. Angioblasts are differentiated into hemangioblasts, which further differentiate into two cell types, hematopoietic and endothelial precursor cells. Hematopoietic cells are the early form of all the blood cells whereas endothelial precursor cells differentiate into mature endothelial cells (EC) forming the vessel structure. A number of factors, such as VEGF, vascular endothelial growth factor receptor -2 (VEGFR 2), PDGFs, and FGFs are involved in different stages of the development by inducing the vessel growth. Some factors, such as VEGFR-1, act as stabilizers of the vessel growth [Carmeliet 2000].

2.1.2 Angiogenesis

Angiogenesis is defined as vascular formation from already existing vessels. During embryonic development it is a normal phase of the vascular development. Vessels are dilated, extracellular matrix becomes looser, and permeability of vessel wall increases to enable ECs to migrate and proliferate. Eventually they sprout and form new vessel lumens [Conway et al. 2001].

In adults, physiological angiogenesis occurs only, for instance, during wound healing [Morgan and Nigam 2013] and thickening of endometrium [Rogers et al. 2009]. Pathological angiogenesis might occur as a reaction to hypoxia in an obstructed artery or as a result of mutations in a malignant tumour [Robbins and Cotran 2005].

When ECs are exposed to hypoxia, nitric oxide (NO) is released causing vasodilation as the first step of the process. This induces release of hypoxia-induced factors, such as VEGFs, FGFs, angiopoietin (Ang) -2, and chemokines. As a result, the vessel permeability is increased and intracellular junctions become loosened. Plasma proteins extravasate into the site and create a temporary support structure to enable activated ECs to migrate into the extracellular matrix (ECM) [Carmeliet 2000]. Ang-1 has a function as a stabilizer of the vessel during this process preventing excessive permeability and leakage through the vessel wall [Fagiani and Christofori 2013].

A large number of agents and factors are involved in the process. In addition to VEGFs, FGFs and VEGF-receptors, NOTCH-ligands, DLL4 and placental growth factor (PlGF)

amongst others are presented in the degradation of ECM, migration of ECs and development of new lumen with stabilized endothelial and pericyte layers [Carmeliet and Jain 2012]. However, these vessels lack smooth muscle cells (SMCs), which make them more fragile and less able to adjust to changes in blood flow than the vessels from which they sprout. Blood perfusion through the lumen is essential for the newly formed vessels and regression occurs if a constant blood flow fails to be maintained [Buschmann and Schaper 1999].

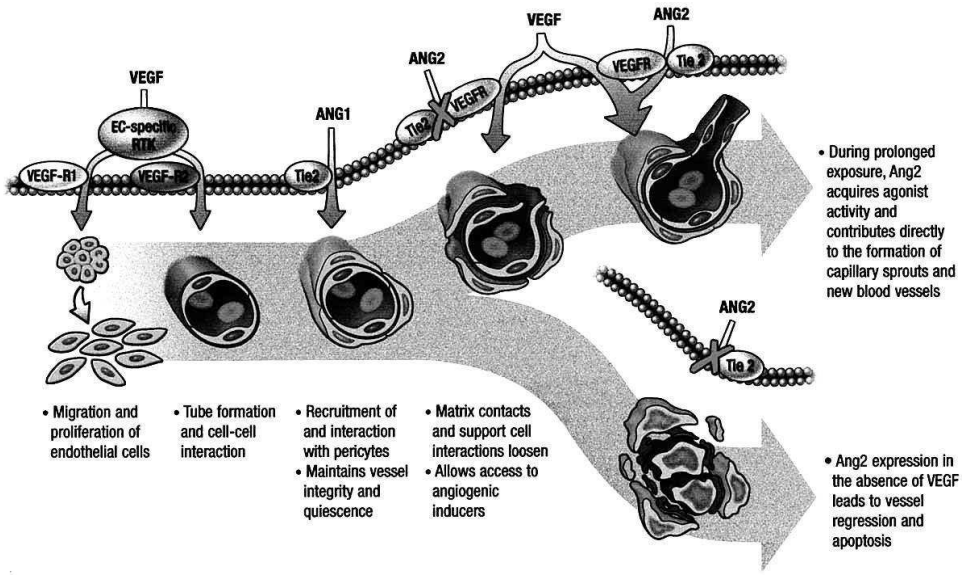

Figure 1 Angiogenesis. Reproduced by permission from Wolters Kluver Health, (Fam et al.), Copyright (2003).

2.1.3 Arteriogenesis

Arteriogenesis is the stage after angiogenesis when a layer of SMCs and pericytes developes around the newly formed endothelium. Arteriogenesis gives vessel its visco-elastic properties and is therefore essential for the proper function and survival of the vessel. It enables the vessel to maintain a stabilized structure under shear stress caused by blood flow [Helisch and Schaper 2010].

During embryonic development, SMCs of coronary arteries originate from atrial epicardium, and SMCs of larger arteries originate from neural stem cells. Formation of SMCs is regulated by a number of factors, such as genes of MEF2 and GATA families. Ang-1 and Tie2 stabilize the wall and induce sprouting and remodeling of the arteries. Transforming growth factor (TGF)-β1 and TGF-βR2 on the other hand inhibit the proliferation and migration of ECs.

In hypoxia-induced arteriogenesis, blood flow increases in collateral vessels surrounding the obstructed artery. This causes vessels to dilate and ECs to produce monokines and monocyte adhesion molecules. Monocytes migrate into the media layer, which triggers the production of FGFs, PDGFs and TGF-β1. As a result, SMC layer thickens and the collateral vessels strengthen and form mature arterial structure [Carmeliet 2000].

2.2 ATHEROSCLEROSIS

Atherosclerosis is characterized by accumulation of fatty plaques inside the arterial wall causing inflammation and obliteration of the artery lumen. Due to a number of genetic and environmental risk factors, accumulation of excess cholesterol along with inflammatory cells in the artery wall cause lumen of the artery to diminish in diameter. This process is slow and happens usually over a course of several years or decades. As the size of the occlusion or stenosis reaches a point where a significant percentage of the artery lumen is obstructed, the tissues' demand of oxygen exceeds the supply that can be delivered around the obstructed site. This leads to ischemia in the target tissue causing symptoms, such as angina pectoris in CAD and claudication in PAD. Development of atherosclerosis takes years to reach a clinically manifested state [Robbins and Cotran 2005]. Genetic background is a risk factor for atherosclerosis, but environmental factors have a substantial role in the progression and prognosis of the disease. Smoking, obesity, dyslipidaemia, diabetes, hypertension, and age along with family history are known risk factors for atherosclerosis [Wilson et al. 1998;Khot et al. 2003]. The incidence of atherosclerosis is higher in men, since women with higher levels of estrogen hormone are better protected against it. However, the incidence in women increases after menopause [Matthews et al. 1998].

Due to these risk factors there is an imbalance in the amount of inflammatory agents and lipids in the coronary blood stream. Lipids, mainly low-density lipoprotein (LDL)-cholesterol, accumulate inside the intimal layer of the artery wall where they form fatty strikes as the early manifestation of atherosclerosis. Fatty strikes are seen as early as in adolescence and young adulthood in the coronary arteries [Ylä-Herttuala et al. 1987]. They consist mainly of T-cells and macrophages [Stary et al. 1994]. At the presence of persistent dyslipidaemia and accumulation of LDL inside the intima, elevated blood pressure, hyperglycaemia, and impaired function of adipose tissue due to obesity lead to oxidation of LDL and dysfunction of endothelial cells. This attracts inflammatory cells, such as macrophages, T-cells, cytokines and interleukins. Inflammatory cells migrate inside the intima layer where macrophages start phagocytosis of fatty cells and turn eventually into fat containing foam cells (Figure 2). This induces chronic inflammation and formation of atheroma inside the artery wall [Hansson 2005]. Over time, the atheroma grows resulting

in narrowing of the artery, i.e., stenosis. In some cases, atheroma remains inside the vessel wall growing towards the outside of the wall and thus may not cause a significant arterial stenosis and classic symptoms [Hackett et al. 1988]. A thin and fragile fibrotic cap covers the surface of the atheroma and is in high risk to erupt. Acute coronary syndrome (ACS), stroke or critical limb ischemia (CLI) may occur at the event of rupture of an atherosclerotic plaque, which causes the intimal core to burst into the arterial lumen. The vessel damage quickly triggers a coagulation cascade involving platelet and fibrin activation, which leads to partial or complete obstruction of the artery (Figure 3) [Libby and Theroux 2005].

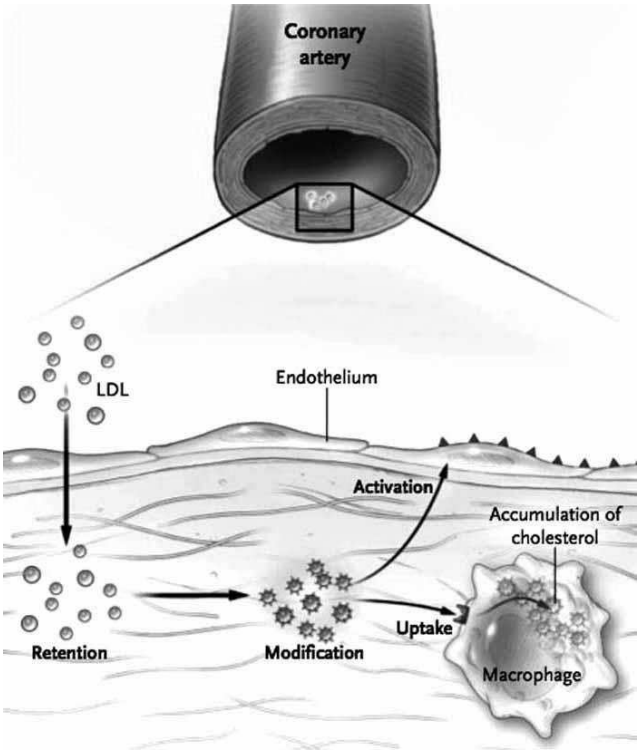

Figure 2 Migration and activation of inflammation in the arterial wall. Reproduced with permission from (Hansson 2005), Copyright Massachusetts Med Society.

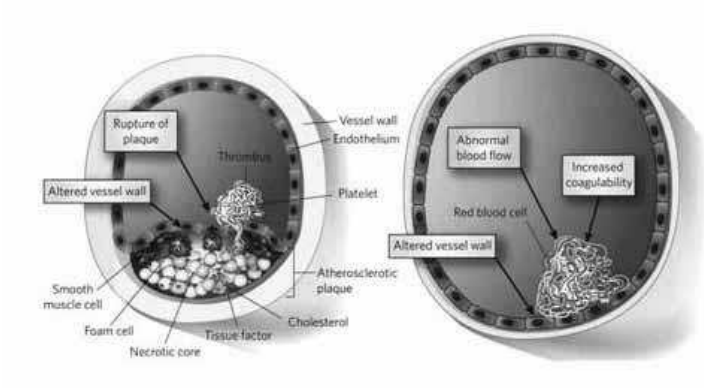

Figure 3 Atherosclerotic plaque rupture. Reprinted by permission from Macmillan Publisher Ltd: [NATURE] (Mackman), Copyright (2008).

2.3 CORONARY ARTERY DISEASE

2.3.1 General aspects

CAD amongst other cardiovascular diseases is one of the most significant causes of morbidity and mortality around the world [Celermajer et al. 2012]. It is a condition where coronary arteries are obliterated or obstructed due to atherosclerosis causing decreased blood flow and insufficient oxygen supply to myocardium [Robbins and Cotran 2005]. As the myocardial oxygen demand increases during physical stress, exercise related symptoms are most commonly the first to appear. In a severe case, it might lead to acute plaque disruption and total obstruction of the artery causing myocardial infarction (MI) [Braunwald 2007]. Diagnosis of CAD is initially based on clinical symptoms supported by risk factor evaluation. Bicycle exercise test (BET), computed tomography of coronary arteries, myocardial perfusion scintigraphy, or coronary angiography can be used to confirm the diagnosis and evaluate the stage of the disease and directions of appropriate treatment [Fox et al. 2006].

The severity of symptoms can be assessed by Canadian Cardiovascular Society (CCS) classification. It is an international classification based on exercise tolerance helping to assess the severity of the disease and the decision of the treatment (Table 1).

Table 1 Canadian Cardiovascular Society (CCS) classification. Modified from *Campeau 1976.*

Stage	Symptoms
CCS1	Ordinary physical activity does not cause angina, such as walking and climbing stairs. Angina with strenuous or rapid or prolonged exertion at work or recreation
CCS2	Slight limitation of ordinary activity. Walking or climbing stairs rapidly, walking uphill, walking or stair climbing after meals or in cold, or in wind or in emotional stress, or only during the few hours after awakening. Walking more than two blocks on the level and climbing more than one flight of ordinary stairs at a normal pace and in normal conditions
CCS3	Marked limitation of ordinary physical activity. Walking one or two blocks on the level and climbing one flight of stairs in normal conditions and at normal pace
CCS4	Inability to carry on any physical activity without discomfort, anginal syndrome may be present at rest

2.3.2 Treatment

Risk factor management

Treatment of CAD varies depending on risk factors, severity of the disease and symptoms as well as concomitant diseases. Managing the risk factors is the foundation of treatment in both primary and secondary prevention of CAD. Cessation of smoking, good control of blood pressure, cholesterol and blood glucose levels have a key role in successful treatment. Obeying dietary guidelines and adequate amount of physical exercise are an essential part of successful treatment and primary prevention, but pharmacological therapy is often required to enhance the effect in particular in the secondary prevention [Fox et al. 2006].

Pharmacological therapy

According to the European Society of Cardiology (ESC) guidelines, antiplatelet therapy is recommended generally to all patients with diagnosed ischemic heart disease. Acetylsalisylic acid (ASA) is prescribed for most patients. However, they are not suitable for patients with a high risk of bleeding complications *Antithrombotic Trialists' Collaboration 2002]. In addition, clopidogrel and ASA prevent stent thrombosis and restenosis after percutaneous coronary intervention (PCI) and stenting [Steinhubl et al. 2002]. Also, the newer antithrombotic agents, prasugrel and ticagrelor, have recently shown to be more efficient in prevention of new cardiac events after PCI compared to clopidogrel [Clemmens et al. 2013]. Beta-blockers have been shown to reduce mortality in secondary prevention in CAD patients after MI. In addition, they help to lower blood pressure and alleviate symptoms [Freemantle et al. 2002]. However, the role of beta-

blockers has been recently challenged. In a trial that included over 44 708 patients, the use of beta-blockers did not reduce the risk of cardiovascular events [Bangalore et al. 2013]. Statins are the most commonly used lipid-lowering drugs and have been proven to efficiently improve prognosis in both primary and secondary prevention [Grundy et al. 2004]. Short or long-acting nitro glycerines alleviate angina symptoms through transient dilation of vessels and reduced diastolic filling of the heart and are used sporadically or regularly in symptomatic patients. However, their use does not have effect on prognosis [Fox et al. 2006]. Calcium channel blockers are efficient in lowering blood pressure and reduce angina symptoms. Additionally they have shown proof of improved prognosis in some CAD patients [Costanzo et al. 2009]. Hyperglycaemia increases the risk of coronary events. In a recent study, patients with type-2 diabetes or impaired fasting glucose had an increased risk of sudden cardiac death compared to healthy controls. Thus, management of diabetes with oral and/or insulin treatment is crucial [Laukkanen et al. 2012].

Exercise training

In addition to pharmacological therapy, exercise training has been shown to reduce mortality and angina symptoms in CAD patients through reduction of risk factors and improvement of endothelial function [Wienbergen and Hambrecht 2013].

Revascularization

In case of inadequate response to pharmacological therapy or ACS, invasive revascularization is required. PCI is defined as a management of coronary artery occlusion by any of various catheter-based techniques, such as percutaneous transluminal coronary angioplasty, atherectomy, angioplasty using the excimer laser, and implementation of coronary stents and related devices [Smith et al. 2001]. PCI was first introduced in 1977 and has since then become the most common invasive treatment method over coronary-artery bypass grafting (CABG). It is especially suitable for patients with one or two vessel disease and it has significantly fewer risks compared to CABG [Serruys et al. 2009]. Stenting has been shown to prevent restenosis compared to sole balloon angioplasty [Al Suwaidi et al. 2004]. Furthermore, newer drug-eluting stents appear to prevent in-stent restenosis more efficiently compared to bare metallic stents in some patient groups [Park et al. 2005; Sousa et al. 2003]. Equally drug-eluting balloons have proven effective in patients with challenging anatomy of coronaries or diffuse calcification [Waksman et al. 2009]. The overall risk of death in a routine procedure is estimated 0.3-1% [Fox et al. 2006]. Common complications are procedure related vascular injuries, bleeding complications and stent thrombosis [Piper et al. 2003; Cheneau et al. 2003].

CABG is the oldest method of revascularization. It is an open heart operation where patient's own veins, usually the saphenous vein, are used as grafts to conduct blood flow around obstructed coronaries to preserve oxygen supply to the hypoxic myocardium [Sabiston and Spencer 2005]. Despite the improved PCI techniques, CABG is still the recommended treatment for patients with a 3-vessel disease or in case of stenosis of the left main coronary artery. It has been shown to reduce 1-year mortality in these groups of patients compared to PCI [Serruys et al. 2009]. Common complications related to CABG are postoperative bleeding, renal dysfunction, arrhythmias, and wound infections among others. Concomitant diseases may also increase the risk of complications. The overall operative mortality is estimated 3% [Sabiston and Spencer 2005].

Neurostimulation

Neurostimulation therapy is used in patients with refractory angina when maximal medical therapy and revascularization options are either insufficient or contraindicated. There are two forms of stimulation being used: transcutaneous electrical nerve stimulation (TENS) and spinal cord stimulation (SCS). The effect of neurostimulation is assumed to be based on the gate theory of pain, where the stimulation of pain is masked by a counterstimulus [Murray et al. 2000]. Other theories to explain the pain reducing effect, such as release of endogenous endorphins and increase in coronary blood flow, have been presented [Chauhan et al. 1994; Eliasson et al. 1998].

Neurostimulation is also suggested to reduce ischemia through prevention of distress and activation of sympathetic nervous system followed by the sensation of ischemic pain. This in turn worsens the ischemia and symptoms. Alleviation of initial pain could thus prevent this cycle [de Jongste et al. 1994]. Pain is essentially a warning signal and there has been a concern whether neurostimulation masks this signal too efficiently in case of a threatening infarction. However, clinical trials have not supported this hypothesis and neurostimulation treatment has proved to be a safe treatment option [Hautvast et al. 1998; Sanderson et al. 1994].

2.4 PERIPHERAL ARTERY DISEASE

2.4.1. General aspects

PAD is characterized by occlusions and impairment of blood supply to the upper or lower extremities. This can be caused by embolism, thrombosis or vasculitis among others, but the most common cause is atherosclerotic lesions. PAD along with CAD and cerebrovascular disease is one of the most important manifestations of cardiovascular diseases [Braunwald 2007]. Risk factors are mainly the same as with other cardiovascular diseases, including age, diabetes, hypertension, hypercholesterolemia, genetic

background, and smoking [Tendera et al. 2011]. However, smoking seems to have a particular role in pathogenesis of PAD compared to CAD, which makes it a remarkable single risk factor [Fowkes et al. 2000; Ingolfsson et al. 1994]. The incidence of PAD increases significantly after the age of 50 and is higher in men than in women [Kroger et al. 2006].

PAD can be classified into different categories based on the severity of symptoms and other clinical manifestations. The Fontaine classification was initially presented in 1954 and is still widely used in clinical practice (Table 2) [Fontaine et al. 1954]. Additionally Rutherford's classification is used alongside *Tendera et al 2011+. Other, more objective means measuring hemodynamic functions, such as ankle-brachial index (ABI) and transcutaneous oxygen pressure ($TcOP_2$), have been introduced [Aronow 2012]. Magnetic resonance angiography (MRA), computed tomography angiography (CTA), and in some cases ultrasound and treadmill exercise tests are equally used in diagnostics [Tendera et al. 2011].

Intermittent claudication is usually the first symptom of PAD. At a progressed stage it might lead to CLI in which case the distal perfusion pressure and nutrient blood flow to the diseased limb are severely disturbed by macrovascular lesions. Rest pain, chronic wounds and/or gangrene are present at this stage [Becker et al. 2011]. Acute limb ischemia (ALI) is characterized by rapid onset and progression of ischemic symptoms and can be caused by acute thrombus or embolus. Emergency revascularization is often needed [Tendera et al. 2011].

Table 2 Fontaine classification. Modified from *Tendera et al. 2011.*

Stage	**Symptoms**
Stage I	Asymptomatic
Stage IIa	Mild claudication
Stage III	Ischaemia rest pain
Stage IV	Ulceration or gangrene

2.4.2 Treatment

Risk factor management

Proper management of risk factors is the foundation of the treatment in patients with diagnosed PAD. Lowering high blood pressure and cholesterol as well as high blood glucose are an essential part of the treatment [Mancia et al. 2009; Collins et al. 2003; Jude et al. 2010]. Smoking has been shown to be the most important individual risk factor for PAD [Diehm et al. 2011]. Therefore cessation of smoking has a substantial impact on the outcome of clinical symptoms such as claudication and peripheral ulcers. Current or

previous smoking increases the risk of PAD in lower extremities [Ness et al. 2000; Tendera et al 2011].

Pharmacological therapy

Anti-platelet medication is part of the standard treatment in all PAD patients. ASA is most commonly used and reduces the risk of cardiovascular events and mortality. In addition, clopidogrel reduces the risk of adverse events. However, anti-platelet treatment has not been shown to significantly affect the walking distance [Momsen et al. 2009]. Use of beta-blockers does not have a negative outcome regarding claudication and is indicated in the presence of simultaneous CAD [Poldermans et al. 2009].

Exercise training

Regular walking exercise has been proved to reduce intermittent claudication symptoms and slow progression of the disease [Bendemacher et al. 2006]. Set exercise treatment (SET) improved the exercise tolerance and walking distance by 50-200% [Watson et al. 2008]. However, this form of treatment is not suitable for patients with CLI since it might worsen already existing wound lesions or gangrenes [Diehm et al. 2011].

Revascularization

Revascularization is considered in case of CLI, ALI or insufficient response to medical therapy in patients with intermittent claudication. Endovascular treatment is less invasive compared to surgery and thus makes revascularization an option to a larger number of patients [Tendera et al. 2011]. In addition, mortality and risk of complications are lower compared to surgery and therefore percutaneous transluminal angioplasty (PTA) is commonly the primary revascularization option [Weinberg et al. 2011]. Drug-eluting stents and balloons are equally used in PTA to reduce the risk of restenosis [Buechel et al. 2012]. Bypass surgery or endarterectomy are considered in case of diffuse and advanced disease. Surgical options also offer more sustainable results and a longer lasting conservation of blood circulation [Tendera et al. 2011].

Amputation is the final surgical option in case of irreversible ischemia and necrosis and if other forms of treatment are unsuitable or insufficient. Prognosis with amputated patients is generally poor and a two-year mortality rate after below-knee amputation is up to 30% [Norgren et al. 2007].

Neurostimulation

Neurostimulation is not as commonly used in treatment of PAD as it is in patients with angina pectoris, although it is indicated as a treatment of PAD related claudication and

chronic pain of extremities [Rokyta and Fricová 2012]. Additionally, SCS has been successfully used in treatment of Buerger's disease related claudication, which is a chronic vascular inflammatory disease causing similar ischemic symptoms and outcomes as PAD. Patients in the case report gained long-term benefit from the treatment [Vasquer Quiles et al. 2009].

2.5 GENE THERAPY

2.5.1 Vectors

Viral vectors

Growth factors used in gene therapy cannot be transferred into the target cell without an efficient and safe delivery vector. A vector is required for the gene to pass the cell membrane and for successful expression of the transgene. Vectors used in gene therapy can be divided into two main categories, viral and non-viral vectors. Viruses are the most commonly used vectors due to their natural ability to penetrate and infect cells. This property also makes them more efficient in terms of transduction and leads to a higher gene expression compared to non-viral vectors [Giacca and Zacchigna 2012; Wang et al. 2012].

Replication deficient adenoviruses are the most widely used gene delivery method of all the viral vectors in preclinical and clinical trials [Patel et al. 1999; Whitlock et al. 2004]. Over a hundred different types of adenoviruses are known to date and they are common pathogens causing infections of respiratory and gastrointestinal tract [Cupelli and Stehle 2011]. Adenoviruses are DNA viruses and their benefits as gene therapy vectors are naturally high transduction efficiency to non-replicating cells and high gene expression. Adenoviruses have a theoretical ability to integrate into the host genome and thus cause potential mutagenesis. However, probability of such integration has been considered low [Harui et al. 1999]. First adenoviruses used in gene therapy were engineered to be replication deficient by deleting certain parts of its genome (E1 and E3). However, sections of the genome capable of triggering immune responses and theoretically mutagenesis in host cell remained [Danthinne and Imperiale 2000]. These properties caused safety concerns. In second and third generation adenoviral vectors inflammation triggering properties have been targeted [Giacca et al. 2012]. These vectors are often referred to as "gutless", since the genome is replaced with the DNA of the therapeutic agent and viral genome does not become expressed. This reduces the risk and severity of host immune response, but the viral capsule alone is capable of triggering inflammation and thus the risk has not been entirely eliminated. However, safety has improved since the introduction of these third generation vectors [Alba et al. 2005; Räty et al. 2008]. Adenoviruses have also

other applications in biomedicine and in addition to gene therapy their use as oncolytic agents is increasing and promising results have been achieved [Ganly et al. 2000; Cerullo et al. 2012].

Retroviruses were the first vectors used in gene therapy. Retrovirus is a ribonucleic acid (RNA) virus, which after transduction into the host cell, is reversed into DNA and becomes integrated as part of the host genome [Gaffney et al. 2007]. Retroviruses very efficiently transduce proliferating cells, however, their ability to enter non-mitotic cells, such as cardiac myocytes or ECs is poor and therefore they are not particularly useful in cardiovascular gene therapy. Although retroviruses have been engineered and made replication deficient [Wu et al. 2005], a potential risk of mutagenesis into a replication capable virus, and carsinogenesis due to its integration into the host genome, cannot be excluded [Manilla et al. 2005].

Lentiviruses are a subgroup of retroviruses and use similar mechanism to transduce target cells as retroviruses. They are based on human immunodeficiency (HI)-1 virus and differ from other retroviruses with their ability to transduce non-replicating cells [Giacca et al. 2012]. Nevertheless, they share the same risks and problems with other forms of retroviruses. Due to advancements in biotechnology and vector engineering, safety profile of lentiviruses has improved and third generation lentiviruses currently used in trials have demonstrated a better safety profile regarding immune responses and potential mutagenesis. They are used in preclinical and clinical trials for a variety of genetic disorders, such as sickle cell anaemia *Pestina et al. 2009+, β-thalassemia [Cavazzana-Calvo et al. 2010; Miccio et al. 2008] and adrenoleukodystrophy (ALD) [Cartier et al. 2009].

Adenoassociated viral (AAV) vectors belong to the family of *parvoviridae* –viruses and they are the smallest viruses used in gene therapy. Currently 12 different serotypes of AAV have been identified and characterized. AAV2 serotype is the most frequently used in gene therapy [Giacca and Zacchigna 2012]. AAVs bind to several different receptors, such as heparin sulphate proteoglycans (HSPGs), avß5 integrin and fibroblast growth factor receptor (FGFR)-1 [Zentilin and Giacca 2008]. Advances made in vector engineering in the past few years have significantly improved AAVs properties as a delivery vector and the latest generation of AAVs have a number of favourable features. AAVs have a simple structure and no viral proteins are expressed in the target cells. In addition, viral genome does not integrate in the host genome. Thus, the risk of immunological responses and harmful mutations is reduced. AAVs also seem to cause long-term gene expression in the target cells [Büning et al. 2003; Ortolano et al. 2012;]. Due to these properties AAVs have become popular vectors in clinical trials. Some trials have achieved successful results in the treatment of e.g. heart failure, haemophilia B and hereditary blindness [Bainbridge et al. 2008; Giacca and Baker 2011; Kay et al. 2000].

A number of other viruses have been investigated as potential gene delivery mediators. For instance, baculoviruses and herpes simplex virus (HSV) have been used in gene therapy. The former in therapy targeted to the heart, liver and brain [Heikura et al. 2012; Hoare et al. 2005; Lehtolainen et al. 2003] and the latter in particular for cancer and diseases of the central nervous system [Marconi et al. 2008].

Non-viral vectors

The use of non-viral vectors in gene delivery has many potential advantages compared to viral vectors. They are cheaper and easier to produce. More importantly, they do not trigger the host immune system or dispose a similar risk of mutagenesis compared to viral vectors, and are thus safer to use. Non-viral vectors can additionally be administered repeatedly. Despite of these advantages, the main obstacle for their more extensive use is lack of efficiency. In terms of transfection efficiency and gene expression time viruses are superior to non-viral vectors [Wang et al. 2012]. Plasmids are commonly used non-viral vectors in gene therapy [Hedman et al. 2003; Kastrup et al. 2005; Sarkar et al. 2001], but the gene expression time has mostly been limited to 1-2 weeks [Ylä-Herttuala and Alitalo 2003]. They are naturally hydrophilic, which complicates their transduction through lipophilic cell membranes. Studies have also indicated that plasmid vectors injected directly into the nucleus of non-dividing cells cause high gene expression. However, if the delivery only reached cytoplasm, the gene expression turned out to be very weak [Capecchi 1980; Mirzayans et al. 1992]. This is caused by degradation of free DNA bound to plasmid vector by cytoplasmic nucleases after phagocytosis [Dean et al. 2005]. Further studies have shown only 1-15% of the DNA to reach the nucleus and eventually express the gene [Tachibana et al. 2001]. This phenomenon significantly limits the use of plasmid vector in non-dividing cells.

For dividing cells the transduction might be more efficient since the structure of the nucleus brakes and divides during mitosis allowing easier entry for DNA into the nucleus. To solve these problems, different enhancers, such as liposomes and chitosan have been used to add a lipophilic component to the vector and thus facilitate the transduction through the cell membrane [Al-Dosari and Gao 2009]. In addition, other means to enhance transduction, such as affecting cell membrane photochemically or with ultrasound [Kloeckner et al. 2004; Taniyama et al. 2001] and packaging vectors in multifunctional, protective “envelopes” *Nakamura et al. 2006+, have been investigated.

2.5.2 VEGF Family

VEGF-A

VEGF-A is the best known and the most investigated member of the VEGF family. It was cloned for the first time in 1989 [Leung et al. 1989] and since then has been used in numerous preclinical and clinical gene therapy trials. VEGF-A has seven isoforms: 121, 145, 148, 165, 183, 189 and 206. However, three of these isoforms, 121, 165 and 189 code 99% of all the expressed growth factors and thus have been the main focus of interest in gene therapy [Whitlock et al. 2004]. VEGF-A has two main receptors, VEGFR-1 and VEGFR-2. In addition, isoforms 165 and 145 are bound by neuropilin-1 and 2. VEGFR-2 is the most important signalling receptor and in angiogenesis, where as VEGFR-1 may also inhibit angiogenesis [Ylä-Herttuala et al. 2007].

VEGF-A is expressed by ECs as a response to tissue ischemia. It is expressed in all tissues during vascular formation. Along with other growth factors and regulators it induces permeability, migration and proliferation of ECs and is the most important single growth factor in angiogenesis. VEGF-A is also known to participate in vascular homeostasis and maintenance of stabilized vascular structure [Zachary et al. 2000]. VEGF-A knockout mice are embryonic lethal [Karkkainen et al. 2004] and deletion of endothelial VEGF-A in healthy mice caused systemic endothelial apoptosis and degradation leading in severe haemorrhage, intestinal perforations and premature death [Lee et al. 2007]. This further emphasizes the significance of VEGF for healthy functioning vasculature. It has been suggested that VEGF-A is involved in the development of atherosclerosis in animal models due to its overexpression in atherosclerotic arterial wall through promotion of pro-inflammatory agents and pathological function of ECs and SMCs [Inoue et al. 1998;Bräsen et al. 2001]. However, no evidence regarding progression of atherosclerotic plaques has been seen in a large number of VEGF-A trials [Laitinen et al. 1998;Henry et al. 2003;Kastrup et al. 2005]. Thus this question remains unanswered, but it seems that the presence of VEGF-A is rather a consequence of hypoxia than the initial cause of the disease process.

In preclinical trials isoforms 121 and 165 have shown the most promising results in formation of new collateral vessels and increase of tissue perfusion [Rissanen et al. 2005; Perrin et al. 2004; Mack et al. 1998; Magovern et al. 1997]. A clinical trial showed $VEGF_{165}$ to induce collateral circulation and perfusion in patients with critical limb ischemia [Baumgartner et al. 1998]. Furthermore, $VEGF_{165}$-mediated intracoronary gene therapy improved myocardial perfusion in CAD patients [Hedman et al. 2003; Losordo DW 1998].

VEGF-B

VEGF-B is present in angiogenesis, but in lower quantities than VEGF-A, and has also been shown to be expressed faster in hypoxic tissue. It is expressed in heart, skeletal muscle and brown adipose tissue [Robbins and Cotran 2005]. VEGF-B has been considered less useful for proangiogenic therapy and has not gained as great interest in research as VEGF-A and some other growth factors [Ylä-Herttuala et al. 2007]. VEGF-B knockout mice have no major abnormalities [Li et al. 2009]. However, VEGF-B mediated gene therapy was shown to improve myocardial function in pigs [Lähteenvuo et al. 2009] and in mice with heart failure through angiogenesis, cell proliferation and inhibition of apoptosis [Huusko et al. 2012]. In addition, VEGF-B has recently been discovered to have an important role in regulation of trans-endothelial transport of circulating fatty acids into cardiac and skeletal muscles [Hagberg et al. 2010]. Furthermore, reduction in VEGF-B signalling increased insulin sensitivity, preserved pancreatic function and had a favourable outcome on lipid profile in mouse models [Hagberg et al. 2012].

VEGF-C

VEGF-C is mainly expressed during lymphangiogenesis, but also has a role in angiogenesis. Equally it is present during vasculogenesis and development of lymphatic vessels in embryo. VEGF-C knockout mice are embryonic lethal [Karkkainen et al. 2004]. Significance of VEGF-C in therapeutic angiogenesis has been considered less clear and therefore it has not been studied as vigorously as other growth factors for proangiogenic gene therapy. VEGF-C mediated gene therapy has proven to be efficient *in vivo* in formation of new lymphatic vessels and treatment of oedema caused by malfunctioning lymphatic drainage [Saaristo et al. 2002; Enholm et al. 2001]. Similar to VEGF-A, VEGF-C has been suggested to have a role in maintenance and progression of atherosclerotic inflammation. VEGF-C was discovered to be present in arterial intima and macrophages in atheroma plaques of human coronary arteries [Rutanen et al. 2005; Nakano et al. 2005]. However, progression of atheroma lesions has not been confirmed by other VEGF-C studies [Hiltunen et al. 2000; Anisimov et al. 2009]. Furthermore, CD11b(+)- derived VEGF-C was found to enhance tissue perfusion in ischemic murine hind limb [Kuwahara et al. 2012] and to prevent restenosis [Rutanen et al. 2005]. VEGF-C binds to three different receptors, VEGFR-2, VEGFR-3 and neuropilin-2 [Ylä-Herttuala et al. 2007].

VEGF-D

Both VEGF-D and VEGF-C function as a lymphangiogenic growth factors and they share the same receptors,VEGFR-2 and VEGFR-3 [Ylä-Herttuala et al. 2007]. VEGF-D knockout mice have no major abnormalities [Karkkainen et al. 2004]. Two different isoforms of

VEGF-D have been identified, a full-length and a shorter, mature (ΔNΔC) form. VEGF-D is proteolytically activated and does not require hypoxia for induction or regulation. In particular the mature form of VEGF-D, also referred as VEGF-D$^{\Delta N\Delta C}$, has a less aggressive but longer and more sustainable expression compared to other VEGFs [Zachary and Morgan 2011; Rutanen et al. 2004]. The length of gene expression seems to be crucial for the efficiency, and thus VEGF-D$^{\Delta N\Delta C}$ is expected to show improved results regarding collateral vessel growth and tissue perfusion. Various preclinical studies have demonstrated encouraging results. Adenoviral VEGF-D$^{\Delta N\Delta C}$ gene transfer induces microvessel growth in a rabbit hindlimb model where as the full-length VEGF-D was detected mostly to regulate lymphangiogenesis [Rissanen et al. 2003]. Another VEGF-D$^{\Delta N\Delta C}$ study using baculovirus as a vector equally showed evidence of increased vascular perfusion in rabbits skeletal muscle [Heikura et al. 2012]. In porcine heart adenoviral VEGF-D$^{\Delta N\Delta C}$ gene transfer increased myocardial perfusion and induced angiogenesis compared to VEGF-A$_{165}$ [Rutanen et al. 2004]. In addition, increased vessel formation was detected in diabetic rabbit skeletal muscles [Roy et al. 2010].

According to studies performed *in vivo*, VEGF-D seems to have an important role in cancer metastasising via lymphatic circulation through enhancement of lymphangiogenesis and regulation of prostaglandin production [Karnezis et al. 2012]. Furthermore, downregulation of VEGF-D and VEGF-C production and lymphangiogenesis through inhibition of VEGR-3 prevented lymph node metastasis in mouse models [He et al. 2002; Lin et al. 2005].

PlGF

PlGF is a member of the VEGF family and it is expressed in placenta, lungs, and thyroid gland. It has three isoforms, 131, 152, and 203. 131 and 203 are soluble whereas 152 binds to heparin sulfate. PlGF is involved in hypoxia-induced angiogenesis by stimulating production and migration of macrophages. Activation of the immune system promotes release and production of other angiogenic factors. In addition PlGF activates proliferation and migration of ECs and stimulates fibroblasts as well as other mural cells in the vessel wall [Ylä-herttuala et al. 2007]. Furthermore, there is evidence, that PlGF is involved in pathogenesis of diseases affecting the nervous system [Chaballe et al. 2011]. PlGF has many functions in normal vascular development. However, studies *in vivo* have shown PlGF to be redundant in many of these processes and PlGF knockout mice show no abnormalities or disturbance in the development of vasculature and later homeostatic functions. Further studies suggest that the role of PlGF is emphasised during pathological angiogenesis. PlGF has been shown to be overexpressed in pathological processes in heart, retina, skeletal muscle, wound and tissue damage healing, tumour growth, and

haematological malignancies. It has been a target of investigation especially in anti-angiogenic cancer research [Dewerchin et al. 2012].

2.5.3 Other growth factors

PDGF

PDGF is a growth factor that consists of two protein chains, A and B. They can form three different isomers, AA, BB and AB. PDGF C and D have also been discovered. However, their significance is yet to be determined [Li et al. 2003]. PDGFs are involved in growth of connective tissue cells such as fibroblasts and SMCs. PDGFs bind to receptors PDGFR α and β. PDGF is restored in α-granules of thrombocytes and is released during thrombocyte activity. Macrophages, ECs, SMCs, and tumour cells can also express PDGF. Expression is followed by proliferation of SMCs, fibroblasts and monocytes, which enhances angiogenesis [Chen et al. 2012]. PDGF- β prolonged the angiogenic effect in combination with VEGF-A in rabbit hind limb [Korpisalo et al. 2008].

FGF

The family of FGFs has multiple members. To date 22 different members have been discovered. In general they are involved in tissue growth, proliferation, and migration. They act as an inducer in wound healing and tissue repair through activation of macrophages, fibroblasts, and ECs. In embryo, FGFs have a role in the development of skeletal muscles, lungs, blood cells, and bone marrow [Beenken and Mohammadi 2009]. FGF-1 and FGF-2 affect ECs and they have been shown to be involved as pro-angiogenic agents [Ware and Simons 1997; Iwakura et al. 2000]. Activation of a particular gene, PR39, is known to be a trigger for overexpression of VEGF and FGF-2. This has been proven to increase myocardial perfusion in porcine model [Post et al. 2006]. Other FGF members of interest are FGF-4 and FGF-5, which also have shown to improve perfusion and increase left ventricular (LV) function [Rissanen et al. 2003;].

HGF

Hepatocyte growth factor (HGF) originates from mesenchymal cells and it has a mitogenic effect on most epithelial cells such as hepatocytes and epithelial cells of skin and lungs. It binds to tyrosine kinase receptors and thus promotes proliferation and migration of the cells [Lavu et al. 2011]. During embryonic development, HGF acts as a morphogenic agent and promotes scattering and migration of cells [Robbins and Cotran 2005]. HGF gene therapy has been shown to induce angiogenesis [Morishita et al. 2004] as well as decreased scar formation and improved myocardial function after MI in animal models [Li et al. 2003; Azuma et al. 2006].

IGF

Insulin-like growth factor (IGF) is a group of growth factors, which consists of three members, IGF-I, IGF-II, and insulin. These three ligands have partly similar peptide sequences and basic molecular structures. In addition to metabolic functions, IGF-I and IGF-II are also known to have cell proliferation promoting activities [Juul 2003]. IGFs have two main receptors: insulin receptor (IR) and IGF- I receptor (IGF-IR). Both are tyrosine kinase receptors and as names suggest, insulin binds to IR and IGF-I and IGF-II to IGF-IR [LeRoith et al. 1995]. In embryo, IGF-I is assumed to have a role in fetal growth and, along with growth hormone (GH), regulation of placental amino acid transport from mother to fetus. Deletion of IGF-I has shown to result in small size of the fetus in animal models [Liu et al. 1993]. Levels of IGF-I have been shown to increase as pregnancy advances and premature infants were detected to represent with lower levels of IGF-I [Leger et al. 1996]. IGF-I induces collateral vessel growth and therefore higher level of serum IGF-1 has been shown to be related to better survival in CAD patients after acute MI [Lee et al. 1999]. IGFs are involved in cell proliferation and therefore there has been evidence of connection between high circulating levels of IGF-I, IGF-II and cancer risk [Weroha and Haluska 2012; Harman et al. 2000; Kaaks et al. 2002]. Studies have suggested patients with GH deficiency and low IGF-I level to have a diminished overall risk of cancer [Erfurth et al. 2001]. In addition, IGFs have been shown to have a role in a large variety of other diseases affecting, for instance, liver and thyroid gland [Juul 2003].

2.6 CLINICAL VEGF TRIALS

2.6.1 Coronary artery disease

Some of the first nonrandomized VEGF-A trials of cardiovascular diseases used recombinant VEGF-A_{165} protein (RhVEGF-A_{165}) as an angiogenic growth factor. Previous preclinical studies had showed promising results regarding efficiency [Harada et al. 1994]. A clinical trial with 15 patients reported dose-dependent improvement in myocardial perfusion and a good safety profile at lower protein dosages. The main safety issue was hypotension, which occurred at higher VEGF-A concentrations [Hendel et al. 2000].

The VIVA trial was a randomized phase II trial, using intracoronary RhVEGF-A_{165} with adenoviral vector as a study drug. There was no difference in exercise tolerance or angina symptoms between the treatment and placebo groups at 2-month follow-up. However, a statistically significant improvement in the treatment group regarding angina symptoms was detected at 4-month follow-up [Henry et al. 2003]. The results from these trials were considered discouraging and to date no further clinical trials using recombinant VEGF-A have been performed.

One of the first nonrandomized, phase I studies to use VEGF-A_{165} gene transfer in no option CAD patients was performed by Losordo et al. in 1998 via minithoracotomy. The procedure was performed on five patients using adenovirus as a delivery vector. The treatment appeared safe and all patients experienced relief in angina symptoms and improvement in myocardial perfusion was detected [Losordo et al. 1998]. Rosengart et al. investigated the effects of adenoviral VEGF-A_{121} gene therapy in a nonrandomized study that recruited 21 CAD patients. 15 of the patients received VEGF-A_{121} as an adjunct to CABG and six patients received the gene through minithoracotomy. No serious adverse events were reported during a 6-month follow-up. In addition, improvement in exercise tolerance and reduction of angina symptoms were reported [Rosengart et al. 1999]. A long-term safety follow-up of the patient cohort was recently published. The results showed no significant difference in mortality, the incidence of cancer or retinopathy in 11,8-year follow-up [Rosengart et al. 2013].

Several randomized, phase II and III clinical trials using VEGF-A gene as a therapeutic agent for patients with advanced CAD have been conducted (Table 3). The Kuopio Angiogenesis Trial (KAT) recruited a total of 103 patients divided into three groups, two of which received intracoronary VEGF-A_{165} gene transfer either with adenoviral or plasmid/liposome vector and the third, control group received ringer's lactate. Patients were followed up to one year. A significant improvement in myocardial perfusion was detected in AdVEGF-A_{165} group compared to p/lVEGF-A_{165} and control groups. In addition, gene transfer was reported to be well tolerated and feasible. Mild, transient elevation in infection parameters and body temperatures were seen in the advVEGF-A_{165} group [Laitinen et al. 2000; Hedman et al. 2003].

In the NOVA trial, 17 patients were randomized 2:1 to treatment and control groups. The treatment group received intramyocardial injections of VEGF-A_{121} with adenoviral vector. In a one-year follow-up treatment proved to be safe, but no significant improvement in exercise tolerance or symptoms were detected between the groups. The number of study the patients was aimed to be abundantly higher, but the trial was determined prematurely [Kastrup et al. 2011].

Adenovirus mediated VEGF-A_{121} gene transfer was equally used in the REVASC trial. The REVASC trial recruited 67 patients randomized into treatment and control groups. Intramyocardial gene transfer was performed through minithoracotomy. The trial showed significantly improved exercise tolerance in the treatment group 26 weeks after the transfer although significant difference in the ST-levels during exercise test was not reported. In addition, there was a transient improvement in angina symptoms in VEGF-treated patients compared to the control group. There was no difference in the number of adverse events between the groups [Stewart et al. 2006].

In the Euroinject I, a controlled and randomized phase II trial, 40 patients received percutaneuous intramyocardial injection of VEGF-A_{165} with naked plasmid vector. Placebo plasmid was used for the 40 control patients. No significant safety concerns were reported. Although no significant improvement in stress-induced myocardial perfusion was reported, some enhancement was seen in regional wall motion [Kastrup et al. 2005].

The Northern trial used VEGF-A_{121} and plasmid vector mediated gene transfer administed percutaneously to myocardium. A total of 93 patients participated in the study. Patients were randomized into the treatment (n=48) and control groups (n=45). During the 6-month follow-up no improvement in myocardial perfusion was seen in the treatment group compared to the control group. No safety concerns were raised during the trial [Stewart et al. 2009].

Intramyocardial VEGF-C gene transfer with naked plasmid vector failed to show efficacy at an interim analysis in the Genasis trial and was stopped prematurely and unpublished [Zachary and Morgan 2011].

2.6.2 Peripheral artery disease

Patients with severe PAD are a potential target for proangiogenic treatment. New alternative treatment methods are needed for patients that are not suitable for operative treatment. A number of clinical VEGF trials for PAD patients have been conducted.

In a phase I, nonrandomized clinical trial by Baumgartner et al. Nine patients with non-healing ischemic lower limb ulcers and/or rest pain were treated with VEGF-A_{165} gene transfer. The gene was injected into the muscles of the ischemic limb using naked plasmid as a delivery vector. Enhancement in collateral flow in the diseased limb or evidence of improved distal flow was detected in 8 patients. Furthermore, out of seven patients with ischemic ulcers, four were seen to experience significant healing. Transient local tissue oedema was seen in six patients, but no further complications or adverse events were reported [Baumgartner et al. 1998].

In randomized phase II trials VEGF-A_{165} has been used in two separate trials (Table 3). A study by Mäkinen et al. recruited a total of 54 patients with chronic limb ischemia or infrainguinal atherosclerotic occlusions. The patients were randomized into three groups. Two of the groups received VEGF-A_{165} with adenoviral or plasmid/liposome vector and the control group received ringer's lactate. During a one-year follow-up increased vascularity was seen in both treatment groups. Gene transfer was feasible and well tolerated. In another study by Kusumanto et al. 54 diabetic patients with CLI were randomized into VEGF-A_{165} and placebo groups. Plasmid vector was used for gene delivery. No substantial adverse events were reported. Although there was significant difference regarding haemodynamic improvement and ulcer healing to the favour of

VEGF- treated group, there was no difference in the number of amputations between the groups [Kusumanto et al. 2006].

The RAVE trial investigated the effects of local adenoviral VEGF-A_{121} gene transfer compared to placebo in 105 patients with intermittent claudication. No improvement in exercise performance was seen compared to placebo at 12 or 26 weeks. Patients in the treatment group experienced transient dose-dependent tissue oedema. No significant difference in infection markers or other safety parameters were seen between the groups [Rajagopalan et al. 2003].

Table 3 Clinical randomized phase II/III VEGF-A trials for CAD and PAD.

	Trial	Gene	Vector	n	Safety	Efficacy
CAD	KAT	VEGF-A_{165}	Adenovirus or plasmid/liposome	103	Transient immunological responses	Improved myocardial perfusion
	NOVA	VEGF-A_{121}	Adenovirus	17	No safety concerns	No evidence of efficacy
	REVASC	VEGF-A_{121}	Adenovirus	67	No safety concerns	Improved exercise tolerance
	Euroinject I	VEGF-A_{165}	Naked plasmid	74	No safety concerns	Improved myocardial perfusion
	NORTHERN	VEGF-A_{165}	Naked plasmid	93	No safety concerns	No evidence of efficacy
PAD	VEGF peripheral vascular disease trial	VEGF-A_{165}	Adenovirus or plasmid/liposome	54	No safety concerns	Improvement in ulcer healing and haemodynamics
	RAVE	VEGF-A_{121}	Adenovirus	105	Tissue oedema	No evidence of efficacy

2.7 SAFETY AND ETHICS OF GENE THERAPY

2.7.1 Ethical aspects of gene therapy

Gene therapy has raised many ethical questions throughout its history. Some significant adverse events have caused drawbacks and resulted in changes in safety regulations and control regarding trials [Yarborough & Sharp 2009]. However, gene therapy has shown evidence of efficiency and brought hope where other forms of treatment are insufficient or nonexistent [Hacein-Bey-Abina et al. 2010]. In treatment of such conditions, higher risks of adverse events can to a certain extent be tolerated. Furthermore, gene therapy is currently limited to alteration of somatic cells and treatment of germ line cells is not allowed [Kimmelman 2008].

2.7.2 Safety aspects

Cancer

VEGF and other growth factors are known to have an important role in tumour growth [Ferrara et al. 2009]. Due to mutations in growth and proliferation regulating genes, angiogenic growth factors become overexpressed leading to uncontrollable angiogenesis in malignant tumours. This creates a fragile but rapidly growing vasculature inside the tumour providing nutrients for fast dividing cancer cells. Tumour survival is thus dependent on sufficient and functioning vasculature, which has made anti-angiogenic therapy a major focus of interest in cancer research [Kubota 2012]. Angiogenesis is a complex cascade involving numerous different factors and agents that are vital for successful vessel growth. This permits an opportunity to interfere with several different steps of the signalling pathway through either suppression of pro-angiogenic gene expression or enhancement of anti-angiogenic gene expression [Gatson et al. 2012]. Preventing binding of angiogenic growth factors, such as VEGFs and FGFs, to their receptors has proven to be an efficient method to suppress tumour angiogenesis. Some anti-VEGF therapy agents have already been approved for clinical use and have demonstrated a good safety and efficiency profile. For instance, bevacizumab, an anti-VEGF antibody targeting VEGF-A expression, was accepted by the U.S. Food and Drug Administration (FDA) in 2004 for treatment of metastatic colon cancer. In addition, efficient angiogenic inhibition has been investigated. Gene therapy for glioma with angiognesis inhibiting angiostatin reduced angiogenesis and tumour growth in mice [Kirsch et al. 1998]

Acceleration of tumour growth is one of the long-term safety concerns in growth factor gene therapy and the success of anti-VEGF therapy shows its importance in cancer development. There is a theoretical risk related to acceleration of tumour growth and metastasis in patients with underlying or undiagnosed malignancy [Gaffney et al. 2007]. In a preclinical study, where hamster ovarian cells were transfected with VEGF-A_{165} *in vitro*, no increase in proliferation was detected. However, in an experiment by the same group VEGF-expressing cells appeared with an ability to proliferate and develop local tumours *in vivo*. Tumour formation was suggested to be weak and no metastases were detected [Ferrara et al. 1992].

Another potential source of malignancy, other than the gene itself, is the vector used for gene delivery. As discussed earlier, retroviral vectors may cause mutagenesis in the host genome. This effect has been seen in two separate clinical trials treating patients with X-linked severe combined immunodeficiency (SCID-X1) with retroviral gene therapy. The patients lack an interleukin (IL)-2 receptor subunit gamma gene (IL2RG), causing a complete absence of various interleukins necessary for immune defence, T-cells and

natural killer (NK)-cells from birth. This condition usually leads to death in early childhood. Replacement of the missing gene using retroviral vector in the first trial caused four out of nine children to develope an acute T-cell lymphoblastic leukaemia. One of the cases resulted in death [Hacein-Bey-Abina et al. 2008]. For the rest of the patients, including the leukaemia survivors, gene therapy corrected the impaired immune system allowing them to conduct a normal life and development while being exposed to surrounding environmental pathogens [Hacein-Bey-Abina et al. 2010]. The second trial reported using a similar gene and vector caused one patient out of ten to develop leukaemia. Further analyses suggest the mutation to be a consequence of the retroviruses' integration to a certain protooncogene LIM domain only 2 (LMO2). This mutation results in overexpression of the gene and development of leukaemia. Additionally it is possible that the patients had other underlying genetic abnormalities increasing the probability of oncogenesis [Howe et al. 2008].

Short-term safety follow-up studies of clinical cardiovascular VEGF-A gene therapy trials have not demonstrated any evidence of increased incidence of malignancies [Hedman et al. 2003; Kastrup et al. 2005; Henry et al 2003]. However, cancer is in most cases a slowly developing disease and the long-term effects of the treatment can only be evaluated over a period of several years.

Arthritis

Rheumatoid arthritis is an autoimmune inflammatory disease affecting one or multiple joints causing stiffness, pain and in advanced stage destruction of the cartilage and bone structure. Tumour necrosis factor (TNF)-α and different interleukins, e.g. IL-1, IL-2, IL-6, are known as important mediators of inflammation in arthritic lesions [Paleolog 2009]. Inhibition of these cytokines with monoclonal antibodies reduces efficiently disease activity [Maini et al. 1999; Williams et al. 2007].

Angiogenesis stimulating growth factors participate in the development and maintenance of rheumatoid arthritis. TNF-α, IL-1 IL-2 and IL-6 in combination with hypoxia is suggested to induce VEGF-A production, which stimulates angiogenesis in joints and bones [Etherington et al. 2002]. The reinforced vascular structure further maintains and enhances progression of pathological arthritic lesions. There is evidence that the inhibition of TNF-α and IL-1 by using TNF-α antibody reduces expression of VEGF in serum [Paleolog 2009]. In addition, VEGF inhibitors have proven to be efficient in the treatment of arthritis. Anti-VEGF antibody delayed the onset of the disease and reduced swelling and stiffness of joints in mouse models [Lu et al. 2000]. Furthermore, VEGFR-1 alleviated arthritis symptoms in mice [Sone et al. 2001]. Thus, systemic delivery

or leakage of VEGF-A outside its target area may potentially enable development or progression of rheumatoid arthritis [Ylä-herttuala and Alitalo 2003].

Diabetic retinopathy and macular oedema

Diabetic retinopathy is one of the most important causes of loss of vision in the western world [Wang et al. 2012]. Incidence of in particular type 2 diabetes, metabolic syndrome and cardiovascular diseases keep increase constantly all over the world, including developing countries. Despite significant amelioration in the treatment methods of diabetes and its risk factors, the complications remain a major cause of morbidity [Antonetti et al 2012]. It is estimated that over 30% of patients with type 1 or type 2 diabetes develop diabetic retinopathy [Yau et al. 2012].

Persistent hyperglycemia and impairment of endothelial function lead to occlusions and hypoxia of retinal and macular cells which induces production of angiogenic factors such as VEGFs and PDGFs. Overexpression of growth factors activates proliferation and migration of ECs leading to increased vessel permeability and leakage as well as neovascularization. Increase in permeability causes macular oedema, thickening of the central fovea and eventually results in visual impairment [Gardner et al. 2012].

Neovascularization of the retina usually occurs near the obstructed sites where surrounding, perfused vessels suffering from hypoxia begin to loosen endothelial junctions and signalling for proliferation, migration and lumen formation are activated. Advanced retinal neovascularization induces vitreous haemorrhages and detachment of the retina [Antonetti et al. 2012].

Laser therapy was first introduced in 1968 as a treatment for diabetic retinopathy and it has been the treatment of choice for several decades. However, in recent years anti-angiogenic gene therapy targeting VEGF-A signalling has proven its efficiency in the treatment of diabetic retinopathy in both preclinical and clinical studies. Treatment with protein kinase C beta inhibitors reduced macular oedema and prevented vision loss in human clinical trial as well as in pigs and mouse models [Ishii et al. 1996; Danis et al. 1998; Davis et al 2009; Viita et al. 2009].

Successful anti-VEGF-A therapy proves VEGF-A to have a crucial role in the development and maintenance of retinopathy and is thus a safety concern in VEGF-A gene therapy. Local gene delivery and prevention of systemic distribution can help to prevent these adverse effects [Markkanen et al. 2005].

Inflammation

Efficient delivery vectors are an essential part of successful gene therapy. Viral vectors as described above, have shown to have the best transduction efficiency and the longest gene

expression time. However, inflammatory responses related to these vectors have raised concern. Although viruses used in gene therapy have been engineered replication deficient, they contain capsule proteins that may trigger the host immune response [Alba et al. 2005; Ylä-Herttuala et al. 2007]. Safety of viral vectors has improved significantly over the past years and also the risk of serious inflammatory reactions after gene therapy has been reduced [Giacca and Zacchigna 2012].

Perhaps the most significant drawback of gene therapy was encountered in 1999, when an 18-year old patient died after initiation of gene therapy for ornithine transcarbamylase deficiency (OTCD). The cause of death was reported to be a massive immune response with uncontrollable production of cytokines and other inflammatory agents as well as antibodies related to the use of adenoviral vector, followed by a multiple organ failure. The dose of adenovirus give to the patient was very high. Patients recruited in the same trial and treated with the same vector and gene had also shown mild signs of inflammation, such as fever, flu-like symptoms and elevation of infection parameters as well as liver transaminases [Raper et al 2003]. The initial cause of the uncontrollable reaction to adenovirus remained undetermined. There had been very few clinical gene therapy trials prior to the OTCD trial and results gained in previous preclinical studies had shown promising results and a good safety profile [Wilson 2009; Stratford-Perricaudet et al. 1990; Nunes et al. 1999].

Since this incident gene therapy has been carefully re-evaluated and repeated in multiple animal and human studies with different types of vectors and growth factors, but similar fierce inflammatory reactions have not been reported [Zhang et al. 2002; Ishii et al. 2004; Mäkinen et al. 2002; Kastrup et al. 2005]. On the other hand, mild and transient immune responses, such as elevation in body temperature and inflammatory parameters have been shown to be more common in the treatment groups receiving gene therapy via viral vectors in comparison to placebo or non-viral vectors [Hedman et al. 2003]. However, inflammatory reactions have also been reported in placebo patients, and therefore it seems that gene delivery procedure itself might be responsible for this as well [Stewart et al. 2006].

Oedema

VEGF induces cell proliferation and migration of ECs in hypoxic arteries. To enable this, existing vessel structures need to be modified and cell junctions loosened, which leads to increased permeability in the vessel wall and causes leakage into the extracellular space [Alitalo and Adams 2007]. This effect is common in particular in VEGF-A treated PAD patients. Trials have demonstrated an increased oedema shortly after the gene delivery at the treatment site. The patients have been affected by local discomfort and/or pain, but

symptoms have resolved within a few days and no further complications have been reported [Baumgartner et al. 1998; Mäkinen et al. 2002; Isner et al 1996].

Tissue oedema is a far more substantial risk in VEGF-A treated CAD patients. Increased permeability and tissue oedema in the heart may potentially lead to pericardial effusion and cardiac tamponade. To reduce the risk, gene is injected in small amounts to multiple different sites by using the highest tolerated dosage. Despite of minimal effusion [Rajagopalan 2003], serious complications or tamponade have not been reported related to VEGF gene therapy trials.

3 Aims of the study

Aims of this study were to investigate the following outcomes:

1. Long-term safety of local VEGF-A gene therapy in CAD and PAD patients.

2. Long-term efficiency and incidence of cardiovascular events in CAD and PAD patients following VEGF-A gene therapy.

3. Procedural and short-term safety of novel AdVEGF-D$^{\Delta N \Delta C}$ gene therapy in no-option CAD patients.

4 Methods

4.1 VECTORS AND GENE TRANSFER

The patients in the long-term safety studies were treated with CMV-VEGF-A_{165} with Ad or P/L vector. Adenoviruses used in the study were replication-deficient E1-E3- deleted viruses (serotype 5) that were produced in 293 cells by using GMP production methods. Viruses were tested for microbiological or endotoxin contaminations (<20 EU/dose, Whittaker, USA) and for absence of replication-competent viruses. To manufacture the P/L vectors, 1000μl DOTMA: DOPE (1:1) liposomes and 2000 μg cytomegalovirus (CMV)-VEGF-A_{165} containing plasmids were complexed and mixed with Ringer's solution. The plasmids were prepared under GMP and were tested to be free of any microbiological or endotoxin contamination (<200 EU/dose, Whittaker, USA). The vectors were manufactured and provided by Ark Therapeutics Oy.

Intracoronary application of the gene was performed by using an infusion perfusion catheter (Dispatch, diameter 2.5–3.5 mm, length 20 mm; Boston Scientific, MA, USA), after a standard PCI. Immediately after balloon dilatation the catheter was introduced via femoral artery and gene transfer was performed at the site of angioplasty prior to the stent implantation.

In the intramuscular gene transfer of the lower limb, a selective DSA (Siemens Polytron Top, Erlangen, Germany) and intravascular ultrasound (IVUS) (Sonos Intravascular, Hewlett–Packard, Andower, USA) were conducted prior to the PTA. After the PTA procedure gene transfer was done at the same site using an infusion–perfusion coil-balloon catheter (Dispatch, diameter 2.5–3.5 mm, length 20 mm; Boston Scientific, MA, USA) for infrapopliteal lesions and a channeled-balloon catheter (Remedy, Boston Scientific/Medi-tech) for femoropopliteal lesions. The gene transfer was done during a 10-minute infusion (0.5ml/minute), after which the completion angiogram was performed.

In the short-term safety evaluation, the mature form of human VEGF-D with a flag tag (VEGF-$D^{\Delta N \Delta C}$) with a CMV promoter was used. This VEGF-D cDNA lacks N-and C-terminal propeptides and contains only a central VEGF homology domain. An escalating dose of VEGF-D was used for the patients; The lowest titer of $1x10^9$vpu was injected for the four of the first five patients, a titer of $1x10^{10}$ vpu for the next four patients and the last four patients received a titer of $1x10^{11}$ vpu. The three control patients did not receive any intramyocardial injections.

4.2 LONG-TERM FOLLOW-UP OF VEGF-A GENE TRANSFER

4.2.1 Patient selection

The patients for the long-term follow-up studies were initially selected according to specific inclusion and exclusion criteria. Briefly, all CAD patients chosen had stable CCS II or III angina pectoris and a minimum of 60% stenosis in one or two main coronary arteries. No previous coronary interventions were allowed. Exclusion criteria included unstable angina or MI, diabetes and previous malignancies. Women in fertile age were not eligible. In addition, patients with diffuse coronary lesions or complex anatomy of coronaries were excluded.

Patients with PAD were considered suitable if they had an infrainguinal atherosclerotic stenosis suitable for revascularization. Patients with history of malignancies, type 1 diabetes, high PSA or carcinoembryonic antigen (CEA) levels, as well as patients with poor co-operation, fertile women and patients under 50 were excluded [Mäkinen et al. 2002]. Both trials were carried out at the Kuopio University Hospital. All patients in both studies had signed an informed consent, which also included the possibility of investigators to contact the patient for a long-term survey.

4.2.2 Data collection and questionnaire

The patients who had died during the follow-up were identified in both studies either from hospital records or the Finnish Population Register centre. All the other patients were interviewed by telephone or those that could not be reached were sent a questionnaire letter. Questionnaires were formulated for each study. Appendix 1 shows the questions presented for the CAD patients and questions for the PAD patients are presented in the Appendix 2. The standardized questions covered patients' current state of health, occurrence of angina or claudication symptoms and cardiovascular procedures performed. The possible diagnoses of cancer, diabetes or its complications and other diseases were recorded. The causes of death were searched from the hospital or communal health centre records according to the International Classification of Diseases (ICD)-10.

4.3 SHORT-TERM FOLLOW-UP OF ADVEGF-D$^{\Delta N \Delta C}$ GENE TRANFER

4.3.1 Patient selection

In total, 15 patients with CAD not eligible for PCI or CABG ("no-option patients") due to diffuse coronary stenosis, small coronary vessels, repeated revascularizations or too high risk for the operation meeting the study criteria were included in the study.

Their suitability for the study was screened according to the inclusion criteria (Table 4), by clinical examination, laboratory tests, electrocardiogram (ECG), transthoracal echocardiography (TTE) BET and chest x-ray. Patients with type 2 diabetes mellitus

underwent ophtamological examination to exclude pre-existing retinopathy. Concomitant diseases and medication were reported and an informed consent was signed by each patient. The study was approved by the local Ethics Committee and the Finnish Medicines Agency.

The patients were randomized 4:1 to the treatment and control groups. The randomization codes were prepared prior to the start of the study. The randomization codes were given in successive order as each patient was recruited into the study. The randomization codes were covered so that only the operator performing the injections knew the code. The patients and other personnel were blinded for the study drug.

4.3.2 Gene transfer

Endocardial gene transfer was performed under fluoroscopic guidance. A 8.0 F introducer sheath was inserted into the right femoral vein. Transseptal puncture was performed with a transseptal needle 8.5 Fr Agilis™ NxT steerable introducer catheter (St Jude Medical™, St.Paul, Minnesota). After the transseptal puncture unfractionated heparin was given to maintain ACT between 300-350 s. An electroanatomical-mapping and injection catheter (NOGA©, Cordis Corp., Johnson & Johnson company, Miami Lakes, Florida) was introduced into the left ventricle via the transseptal route. Mapping of the left ventricular endocardium was done spot-by-spot to detect the ischemic areas and sites of reduced contraction to evaluate the optimal targets fort the gene injections. NOGA© catheter was used to transduce AdVEGF-D$^{\Delta N \Delta C}$ drug to ten different sites of the most ischemic areas. The injections, in depth 5-6mm, were given to ischemic myocardial areas 5-10 mm apart.

4.3.3 Safety protocol

One day prior to the procedure, patients were admitted to the hospital ward where ECG, vital signs and baseline laboratory parameters were evaluated. On the day of the procedure blood gas analysis and vital signs including blood pressure, heart rate and body temperature, were taken at the time of the gene transfer. ECG, vital signs and laboratory assessment were controlled 4 h after the procedure. Patients were monitored at Cardiac Care Unit (CCU) until the first postoperative morning and were transferred to the cardiology bed ward if no serious adverse events occurred during the first day. The patients were discharged on the second postoperative day if there were no complications.

Laboratory and ECG assessments were repeated on the days 6 and 14. At 90 days patients were invited for a 3-month follow-up visit and clinical signs, laboratory parameters, and ECG were evaluated. To detect potential pericardial effusion, TTE was performed to all patients. In case of no contraindications, patients also underwent MRI at rest and during adenosine infusion.

All adverse events were classified as either serious or non-serious based on the objective definitions of the European Commission Enterprise and Industry Directorate [European commission 2006]. Adverse events were reported regardless of their causality to the study drug. The data was monitored according to the Good Clinical Practice (GCP) guidelines.

Table 4 Inclusion and exclusion criteria, (study III).

Inclusion criteria	**Exclusion criteria**
- Informed consent signed - Age >30 but ≤ 80 years - Severe angina pectoris (CCS II-III) despite of maximal medication - Significant stenoses in coronary angiopraphy - Contraindication to coronary angioplasty or bypass operation (diffuse or distal stenosis, chronic total occlusion, vessels with difficult anatomy, stenosis with severe calcifications, stenosis in small vessels (<2,5mm)) - Angina pectoris or ischemic ST-depression (≥1mm) in the exercise test - LV wall >8mm detected by TTE (treatment area)	- Women in fertile age - Type 1 DM or severe complicated type 2 DM, diabetic retinopathy - Clinically significant anemia (hemoglobin count <120mg/l in male, <110mg/l in female; hematocrite <0.36), leukopenia (b-leukocyte count < $3.0x10^9$/l), leukocytosis (b-leukocyte count >$12.0x10^9$/l) or thrombocytopenia (b-thrombocyte count < $100x10^9$/l) - Renal insufficiency (s-creatinine > 160mg/l) - Liver insufficiency (alanine aminotransferase and s-alcaline phosphatase over 2 x normal) - Haematuria of unknown origin - Severe hypertension (systolic blood pressure >200mmHg or diastolic blood pressure >110mmHg) or significant hypotension (systolic blood pressure <90mmHg) - Significant obesity (BMI >35) - Acute infection - Immunosuppressive medication - Significant impairment of the left ventricular function (EF <25% in TTE or CO< 2l in MRI)

LV=left ventricular; TTE=transthoracal echocardiography; DM=diabetes mellitus

4.4 STATISTICAL ANALYSIS

Statistical analysis was performed by using SPSS statistical software (SPSS 17.0, Chicago, Illinois 60606, USA). Chi-square test was used for categorical variables and linear mixed models for repeated measures. P-value >0.05 was considered significant.

5 Results

5.1 BASELINE CHARACTERISTICS AND PATIENT DEMOGRAPHICS

5.1.1 VEGF-A gene therapy for coronary artery disease

Baseline characteristics of CAD patients at the beginning of the trial are presented in Table 5. There were no statistically significant differences between the study groups. The mean follow-up time was 8.1 years (6.9-9.7 years) and 89 (83%) patients were reached and interviewed for the study.

Table 5 Baseline characteristics at randomization, (study I)

	AdVEGF-A n=37	**P/LVEGF-A** n=28	**Control** n=38	**p-value**
Sex (male/female)	26/11	23/5	30/8	ns
Age (years)	58±8	58±7	56±9	ns
Hypertension	17 (46)	14 (50)	19 (50)	ns
Dyslipidemia	31 (84)	21 (75)	28 (74)	ns
Family history of CAD	21 (57)	16 (57)	25 (66)	ns
Previous myocardial infarction	11 (30)	8 (29)	11 (30)	ns
Previous TIA or stroke	3 (8)	3 (11)	1 (3)	ns
Previous peripheral ASO	3 (8)	3 (11)	1 (3)	ns
Smoker (current/ex)	5/16	4/10	5/15	ns
Acetosalicylic acid	33 (89)	28 (100)	34 (89)	ns
Betablockers	26 (70)	16 (57)	22 (58)	ns
ACE inhibitors	9 (24)	5 (18)	8 (21)	ns
Statins	26 (70)	22 (79)	29 (76)	ns
Calcium channel antagonists	6 (16)	5 (18)	6 (16)	ns
Nitrates	26 (70)	16 (57)	22 (58)	ns

ACE= angiotensin converting enzyme; ASO= atherosclerosis obliterans; CAD= coronary artery disease; TIA= transient ischemic attack; VEGF= vascular endothelial growth. n(percentage)

5.1.2 VEGF-A gene therapy for peripheral artery disease

Baseline patient characteristics at the time of randomization are presented in table 6. The mean follow-up time was 10 (9-12) years. The mean age of patients was 81.9 (62.6-94.7) years and a total of 25 (46%) patients were interviewed for the study.

Table 6 Baseline characteristics at randomization, (study II).

	AdVEGF-A n=18	P/LVEGF-A n=17	Control n=19	p-value
Age (range)	70 (53-86)	74 (55-84)	73 (61-86)	ns
Men / Women	9/9	6 / 11	8/11	ns
Symptoms				
Claudication	14	11	15	ns
Critical ischemia	4	6	4	ns
Coronary artery disease	8	9	13	ns
Previous TIA or stroke	5	5	6	ns
Diabetes	3	4	6	ns
Gene transfer site				
Femoro-popliteal	13	14	15	ns
Infrapopliteal	5	3	4	ns

TIA=Transient ischemic attack. Chi-square test. The values denote n.

5.1.3 AdVEGF-D$^{\Delta N\Delta C}$ gene therapy for coronary artery disease

The baseline characteristics and demographics are presented in the table 7. All recruited patients were men with a mean age of 70.1 years (63-78). All patients had undergone CABG operation in the past and were found to have chronic occlusions or diffuse stenoses in the most recent angiography and suffered from CCS class II/III angina symptoms. Furthermore, revascularization was no longer possible.

5.2. EFFICACY AND LONG-TERM EFFECTS

5.2.1. VEGF-A gene therapy for coronary artery disease

Working ability

Changes in working ability are presented in Table 8. Working ability was assessed 6 months after the gene transfer and at the time of the 8-year follow-up. The overall number of retired patients had increased in all study groups, however, inability to work due to CAD remained low in all groups and no significant difference was detected. The majority of patients had retired at the 8-year follow-up, approximately a third of the patients were still working and only two patients from the AdVEGF-A group were on a sick leave due to CAD.

Table 7 Baseline characteristics at randomization, (study III).

Patient number	Study group	Age	Sex	CCS II/III	Reason for ineligibility	PCI	CABG	Type II diabetes	Family	HTA	Dyslipidemia	Smoking history
1	AdVEGF-D	69	Male	3	Diffuse disease	+	+	+	+	+	+	-
2	AdVEGF-D	66	Male	3	Diffuse disease	+	+	+	+	+	+	+
3	AdVEGF-D	68	Male	3	Diffuse disease	+	+	-	+	+	+	+
4	AdVEGF-D	75	Male	3	Chronic occlusions	+	+	-	-	+	+	+
5	AdVEGF-D	67	Male	3	Diffuse disease	-	+	-	+	+	+	+
6	AdVEGF-D	67	Male	3	Diffuse disease	+	+	+	+	+	+	+
7	AdVEGF-D	78	Male	3	Diffuse disease	+	+	-	+	+	+	+
8	AdVEGF-D	78	Male	2	Diffuse disease	+	+	+	+	-	+	-
9	AdVEGF-D	75	Male	2	Diffuse disease	+	+	-	+	+	+	-
10	AdVEGF-D	75	Male	3	Diffuse disease	+	+	-	+	+	+	+
11	AdVEGF-D	74	Male	3	Chronic occlusions	-	+	+	+	-	+	+
12	AdVEGF-D	63	Male	3	Diffuse disease	-	+	+	+	-	+	-
13	Control	70	Male	3	Diffuse disease	-	+	-	+	-	+	+
14	Control	64	Male	2	Diffuse disease	-	+	+	+	+	+	+
15	Control	71	Male	3	Diffuse disease	+	+	+	+	+	+	+

AdVEGF-D= Adenoviral vascular endothelial growth factor-D; CCS= Canadian cardiovascular society; PCI= percutaneous coronary intervention; CABG coronary artery bypass grafting; HTA= arterial hypertension; + = yes, -=no.

Table 8 Working ability, (study I).

At baseline	**AdVEGF-A** n=32	**P/LVEGF-A** n=28	**Control** n=31	**p-value**
Able to work	23 (72)	17 (66)	21 (68)	ns
Unable to work due to CAD	4 (13)	5 (19)	5 (16)	ns
Retired	5 (15)	4 (15)	5 (16)	ns
At 6 months follow-up				
Able to work	21 (66)	16 (62)	22 (71)	ns
Unable to work due to CAD	1 (3)	3 (12)	2 (6)	ns
Retired	10 (31)	7 (26)	7 (23)	ns
Long-term follow-up				
Able to work	9 (28)	8 (31)	7 (23)	ns
Unable to work due to CAD	2 (6)	0 (0)	0 (0)	ns
Retired	21 (66)	18 (69)	24 (77)	ns

Working ability at baseline and at 6-month and 8-year follow-ups. **CAD=** coronary artery disease; ns= non- significant. n (percentages). Chi-square test.

Exercise tolerance

Exercise tolerance was assessed using CCS classification. At baseline, all patients belonged to CCS classes II or III. Patient distribution is presented in Table 9. At 6-month follow-up the majority of patients (AdVEGF-A 72% vs. P/LVEGF-A85% vs. Control 84%) belonged to the CCS class I and only in total 4 patients to the class III. At the long-term follow-up distribution was somewhat similar between CCS classes I and II. Furthermore, the number of patients in the CCS class III had remained relatively small and in total nine patients belonged to this group.

Table 9 Excercise tolerance, (study I).

	AdVEGF-A n=32	**P/LVEGF-A** n=28	**Control** n=31	**p-value**
At baseline				
CCS 2	16 (50)	13 (50)	19 (61)	ns
CCS 3	16 (50)	13 (50)	12 (39)	ns
At 6 months follow-up				
CCS 1	23 (72)	22 (85)	26 (84)	ns
CCS 2	6 (19)	4 (15)	4 (13)	ns
CCS 3	3 (9)	0 (0)	1 (3)	ns
At long-term follow-up				
CCS 1	14 (43)	12 (46)	18 (58)	ns
CCS 2	13 (41)	12 (46)	11 (35)	ns
CCS 3	5 (16)	2 (8)	2 (7)	ns

Canadian Cardiovascular Society (CCS) classification at baseline, 6 months and at 8-year follow-up (percentages). Chi-square test.

Major cardiovascular events

Cardiac death, ACS, repeated coronary interventions and stroke were considered as major cardiovascular adverse events (MACEs). These events were explored among the CAD

patients during the 8-year follow-up period. Two (2%) cardiovascular deaths occurred during the follow-up: In one patient the death resulted from MI and the other from ruptured aortic aneurysm. Thirteen patients altogether encountered an ACS. One or more angiographies were performed in 21 (24%) patients. PCI and CABG operations were performed in eight (9%) and seven (8%) patients, respectively. The total distribution of MACEs were AdVEGF-A7 vs. P/LVEGF-A 4 vs. Control 9. The difference between the groups was non-significant.

5.2.2. VEGF-A gene therapy for peripheral artery disease

Invasive procedures and amputation

Four patients (7%) had had an amputation of the treated limb (AdVEGF-A 0 vs. P/LVEGF-A 4 vs. Control 1). All of these patients were among the ones who had died and amputations were performed due to non-treatable CLI. In total, 11 (44%) patients had undergone an invasive vascular procedure. Angiography was performed in eight (4 vs. 2 vs. 2), PTA in nine (4 vs. 2 vs. 3) surgical revascularization in two patients (1 vs. 0 vs. 1). The differences between the groups were statistically non-significant.

Other effects

Current PAD related symptoms and exercise tolerance according to Fontaine classification of the interviewed patients were additionally assessed. Exercise tolerance was compared to the baseline class in each group and is presented in Table 10. There were no significant differences between the groups or the two time points within the groups.

Table 10 Exercise tolerance, (study II)

Fontaine class	**AdVEGF-A** n=10		**P/LVEGF-A** n=8		**Control** n=7	
	At randomization	At follow-up	At randomization	At follow-up	At randomization	At follow-up
I	0	2	0	2	0	0
II	7	6	7	6	6	3
III	3	0	0	0	0	3
IV	0	2	1	0	1	1

Severity of ischemic symptoms according to Fontaine classification at randomization and at the 10-year follow-up interview, n=25. p=0.43. Linear mixed models

5.3 SAFETY

5.3.1 Long-term safety of VEGF-A gene therapy

Long-term safety parameters concerning the incidence of overall mortality as well as diabetes and cancer diagnosed after gene transfer are presented in Table 11. The mortality and cancer incidences are presented compared to the initial number of patients recruited in the trials. In comparison to CAD patients, the overall incidence of diabetes and cancer was higher with PAD patients, but there was no significant difference between treatment and control groups in either of the studies. Furthermore, the overall mortality was substantially higher within the PAD patients and approximately half of the patients had

died during the follow-up period, where as mortality in CAD follow-up was 7.5%. No statistical significance was seen between the treatment and control groups. In CAD patients, no cases of diabetic retinopathy or nephropathy were diagnosed. In PAD follow-up, one patient from the VEGF-p/l group had developed a diabetic retinopathy. However, no new cases of nephropathy were diagnosed.

The causes of death were clarified in both studies. In CAD study, malignancy was the cause of death for six patients and the remaining two patients had died of rupture of aortic aneurysm and MI. In PAD patients, the causes of death included malignancy (AdVEGF-A 1 vs. P/LVEGF-A 0 vs. Control 2, p=0.60), cardiac events (1 vs. 2 vs. 2, p=0.68), cerebrovascular events (2 vs. 0 vs. 1, p=0.75), pneumonia (0 vs. 2 vs. 1, p=0.16), PAD related complications (1 vs. 3 vs. 1, p=0.45), COPD 1 vs. 0 vs. 0, p=1.00), Intestinal haemorrhage (1 vs. 0 vs. 0, p=1.00), septic infection (1 vs. 0 vs. 1, p=1.00). For four patients the death certificates were not obtained and the cause of death remained unclear (0 vs. 1 vs. 3).

Table 11 Long-term safety of VEGF-A gene therapy in CAD and PAD patients, (studies I and II).

CAD	**AdVEGF-A** n=37	**P/LVEGF-A** n=26	**Control** n=38	**p-value**
Mortality (all cases)	3/37 (8)	2/26 (7)	3/38 (8)	ns
Cancer (all cases)	1/37 (3)	4/26 (16)	2/38 (5)	ns
Intestinal		2 (8)	1 (2.5)	
Renal		1 (4)		
Skin	1 (3)			
Brain		1 (4)		
Leukemia			1 (2.5)	
Diabetes	2/32 (6)	2/26 (8)	2/31 (6)	ns
PAD	**AdVEGF-A**	**P/LVEGF-A**	**Control**	
Mortality (all cases)	8/18 (44)	8/17 (47)	10/19 (53)	ns
Cancer (all cases)	1/18 (6)	1/17 (6)	3/19 (16)	ns
Gastric			2 (10)	
Skin		1 (6)		
Leukemia	1(6)			
Lung			1(6)	
Diabetes	3/10 (30)	3/8 (38)	2/7 (29)	ns

Incidence of mortality and cancer in the total study population. Incidence of diabetes among the interviewed patients. Chi square test n(percentages); ns=non-significant.

5.3.2 Procedural and short-term safety of AdVEGF-D$^{\Delta N \Delta C}$ gene therapy

Altogether, seven SAEs were reported during the follow-up period. SAEs and related gene doses are presented in Table 12. There were three cases of MI or ACS (AdvVEGF-D 2 vs. Control 1). One of the patients was diagnosed with MI two times during the follow up. One control patient had an iatrogenic pericardial puncture during the procedure. TTEs performed on two following days showed no signs of pericardial tamponation or other clinically significant outcomes. New gene transfer was performed successfully three weeks

later. One patient in the AdVEGF-D$^{\Delta N \Delta C}$ group died 10 days after the gene transfer. In the autopsy, MI was determined to be the cause of death.

One control patient presented with symptoms of respiratory infection on the day of the procedure. Pneumonia was suspected but the confirmative diagnosis remained uncertain. Antibiotic treatment resolved the symptoms within five days and the patient was discharged on the sixth postoperative day. In addition, a patient from the AdVEGF-D$^{\Delta N \Delta C}$ group was diagnosed with pyelonephritis, which was assumed to be a cause of urinary tract catheter.

Mild elevation in C-reactive protein (CRP) levels was detected in all of the patients during hospitalization. No similar changes were seen in blood leukocyte levels. At the 3-month visit, elevated PSA compared to baseline was seen in one patient in the AdVEGF-D$^{\Delta N \Delta C}$ group (3.07-9.17μg/l). Prostate biopsies showed no signs of malignancy or dysplasia. No elevation in the liver function parameters was found (ALT, ALP, LDH) in either of the groups. Clinically non-significant elevation in adenovirus antibody titers was seen at two weeks time in three VEGF-D$^{\Delta N \Delta C}$ patients. None of the patients had serious arrhythmias, such as ventricular or atrial tachycardias or ventricular fibrillation. No sinus arrests or II or III degree AV-blocks were detected during hospitalization.

In total, three patients in the AdVEGF-D$^{\Delta N \Delta C}$ group were detected with mild pericardial effusion (3-6mm). For two of the patients effusion was seen on the second day after the procedure. Additional TTE control was required for one of the patients at one weeks' time point when the effusion had resolved spontaneously. In the third patient, the effusion was seen in TTE at the 3-month follow-up visit. However, the effusion was not detected in MRI performed at the same visit. No further procedures or controls were required.

Table 12 Serious adverse events (SAE) during 3-month follow-up, (study III).

	AdVEGF-D/Dose n=12	**Control n=3**
SAE total	4	3
Death	$1/1x10^{10}$	0
Pneumonia susp.	0	1
Pyelonephritis	$1/1x10^{9}$	0
MI or ACS	$2/1x10^{10}$*	1
Pericardial puncture	0	1

*Both MIs occurred in the same patient.

6 Discussion

Gene therapy has been largely investigated as a novel therapeutic approach especially to hereditary and genetic conditions, but it also offers potential in treatment of chronic and advanced cardiovascular diseases, such as CAD and PAD. VEGF has been shown to enhance vessel growth in ischemic tissues and thus offers new therapeutic possibilities [Rissanen et al. 2005].

Safety is one of the top priorities when investigating and introducing new therapeutic methods. A profound and careful evaluation of risks and adverse effects of these new approaches are essential before permission for wider therapeutic use can be granted. VEGF mediated therapy has theoretical adverse effects that may occur immediately or within a short period of time following the gene transfer and are mainly related to the gene transfer procedure or the delivery vectors. Furthermore, there are processes that may take significantly longer time to develop and cannot be seen until in several years time [Hedman et al. 2011].

The purpose of this work was to further clarify in particular the long-term effects of cardiovascular VEGF-A therapy and provide further evidence regarding some important safety aspects. In addition, the aim was to assess short-term safety of novel AdVEGF-D$^{\Delta N \Delta C}$ gene transfer, which was now used in human clinical trial for the first time. Since the short- and long-term risk factors differ in great deal, the approach and focus of interest in these studies were seemingly different.

6.1 LONG-TERM SAFETY OF VEGF-A GENE THERAPY

Short-term safety of VEGF-A gene therapy is generally well known and has been repeated in a number of clinical trials [Hedman et al. 2003 ;Stewart et al. 2009; Rajagopalan 2003]. The different isoforms of VEGF-A have been used with no significant adverse events. The long-term safety concerns involve among others induction of tumour growth and development of diabetic retinopathy where VEGF is prominently involved in the pathogenesis [Gaffney et al. 2007; Gardner et al. 2012]. However, until now the long-term safety data has been limited. In the 8- and 10- year safety follow-ups we found no difference in mortality or the incidences of cancer, diabetes or its complications compared to randomized control patients. Additionally, in comparison to general population, no significant difference was seen.

Although patients with current or previously diagnosed malignancies were excluded from the trials, there is a theoretical risk of underlying early stage malignant process that

has not been diagnosed. In terms of types of cancers detected, the variation is fairly large and does not seem that any particular type would be overrepresented. Previously, direct causality between gene therapy and malignancy has been shown in trials where children with SCID-X1 syndrome were treated with retroviral gene therapy. In the first trial, four children out of nine and in the second one out of ten were diagnosed with acute T-cell lymphoblastic leukaemia [Hacein-Bey-Abini et al. 2010; Howe et al. 2010]. However, in this study no association with cancer could be seen.

In total, eight new cases of type 2 diabetes were diagnosed in interviewed CAD patients (n=89) and eight in the PAD (n=25) study. This implies that diabetes has a higher incidence within the PAD study population than CAD patients. In addition, overall mortality was higher in the PAD patients. The demographics of the two study populations were somewhat different. In the CAD study, the mean age was lower and many of the patients were still working. The incidence of PAD increases after the age of 60 and the patients with advanced disease are thus older and affected by high incidence of concomitant diseases [Tendera et al. 2011]. In fact, majority of the PAD patients were also diagnosed with CAD. Therefore comparison of the two studies, regarding mortality for instance, is difficult and the results are only observed within each study. In conclusion, VEGF-A gene therapy did not increase the incidence of mortality, cancer, diabetes, or diabetic complications.

6.2 EFFECTS ON EXERCISE TOLERANCE AND MAJOR CARDIOVASCULAR EVENTS

One of the secondary endpoints of this work was to investigate long-term efficiency and number of cardiovascular events and procedures among the patients. There were no significant differences in the number of ACSs or cardiovascular interventions in the CAD study. Additionally, the current exercise tolerance between the groups did not differ from one another. This gives the idea that gene therapy does not appear more efficient compared to control patients. However, it seems that all patients, including the control group, have fewer symptoms in comparison to baseline situation. The placebo effect in gene therapy trials has been claimed to be particularly strong [Lavu et al. 2011;Hedman et al. 2011] and perhaps the results of our study reflect this phenomenon. The number of diagnosed coronary events and interventions however, are more objective measures to assess efficiency. CCS classification is based on patient's own subjective experience of the symptoms, and may be influenced by many other factors affecting exercise tolerance. This study is thus somewhat limited in terms of efficacy analysis. BET and cardiac imaging could provide more reliable data on the efficiency.

In the PAD study, exercise tolerance was equally assessed through a questionnaire. Regarding amputations, there were five patients who had had an amputation of the treated limb, four of which in the plasmid/liposome group. Also 44% of the patients that were interviewed for the study, had had a vascular intervention during the follow-up. There were no statistically significant differences regarding lower limb procedures. However, based on these results, definite conclusions regarding efficacy of the treatment were not drawn due to the small study population and lack of other more objective parameters. In terms of other clinical VEGF trials, the efficiency data is only available from a fairly short period of time [Kastrup et al. 2009; Stewart et al. 2009;Baumgartner et al. 1998;Kusumanto et al.2006]. As of yet, no data with more thorough and objective examinations in order to assess efficacy after several years are available [Lavu et al. 2011]. In the future, this information could be useful to obtain a clearer image of the true effects.

It has been suggested that VEGF is involved in acceleration of atherosclerosis [Isner et al. 2001]. From the safety point of view, the non-significant results regarding cardiovascular events could indicate that VEGF gene therapy does not seem to promote neo-intimal plaque formation or atherosclerosis. In conclusion, VEGF-A gene therapy did not seem to affect the number of cardiovascular events or exercise tolerance. Further objective evaluations are needed to evaluate the long-term efficacy.

6.3 SHORT-TERM SAFETY OF ADVEGF-D$^{\Delta N \Delta C}$ THERAPY

In the short-term safety evaluation of AdVEGF-D$^{\Delta N \Delta C}$ gene transfer the emphasis was on the immediate adverse events and complications related to the study drug, vector or gene transfer procedure. As for the study population, the first fifteen patients from total of thirty patients were included in the interim analysis. All patients recruited were male. The study population is small and definite conclusions on the gender distribution of no-option CAD patients cannot be made. However, it might reflect that there is a higher incidence of CAD in men in younger populations, and thus these patients have time to develop chronic, severe occlusions over the years. Indeed, most of the patients were diagnosed with CAD two or more decades earlier.

In total, seven SAEs were reported in these patients. There was only one death caused by acute MI in the treatment group. In addition, two other patients, one from each group, encountered ACS. Two of these cases occurred in the same patient. All the patients recruited had severe CAD and therefore very high probability of ACS.

There was one case of suspected pneumonia in the control group and one patient in the treatment group was diagnosed with pyelonephritis. Inflammatory reactions have been reported especially related to the use of viral vectors [Ylä-Herttuala et al. 2007], but no correlation to viral or bacterial infections has been seen. All the patients had elevation in

body temperature and CRP level, including the control patients. This indicates that the catheter procedure itself has an effect on the immune system.

Pericardial effusion was detected in three patients in the treatment group. Local tissue oedema has previously been reported in patients having received VEGF-A to lower limb muscles. Also in pig animal model myocardial effusion was detected after intramyocardial VEGF-D gene transfer [Rutanen et al. 2004]. Therefore increased permeability could also be seen after intramyocardial gene transfer in human trial. However, the effusion in all of these patients was minimal and caused no symptoms or need for further procedures other than control TTE. In two of the patients, effusion was seen within two days after the gene transfer where in the third patient the effusion was detected at the 3-month visit. Minimal effusion can be physiological and therefore it cannot be stated with certainty that gene therapy was the initial cause. However, in the further stages of the trial the incidence of pericardial effusion needs to be carefully evaluated.

Transseptal puncture appeared to be feasible to perform and no serious arrhythmias, AV-blocks or sinus arrests were seen during the procedure or during the hospital stay. In later ECG analyses there were no changes compared to the patients' baseline rhythm. One iatrogenic pericardial puncture occured in one control patient, but this caused no further complications and the procedure was later successfully repeated.

None of the patients was reported to have significant changes in blood pressure that would have required further procedures. However, NO- mediated decrease in blood pressure after administration of VEGF has been reported in animal studies [Liu et al. 2001], but this effect was not seen in this study. This could be due to local delivery and relatively small quantity of the gene in order to cause systemic effects that the cardiovascular compensation mechanisms would not be able to manage.

As discussed earlier, malignant processes take years to develop. Theoretically the presence of VEGF could accelerate this process. One of the patients was reported with elevated PSA level compared to baseline three months after the transfer. Biopsies however, showed no signs of dysplasia or malignancy and as the patients were all male with high age, natural causes for elevated PSA could also be present.

Due to the small number of patients, no statistical analysis was performed, since a statistically significant finding could not have been considered reliable and could have potentially led to incorrect conclusions. In order to avoid any misleading results, the findings were reported in a descriptive form. In conclusion, AdVEGF-D$^{\Delta N \Delta C}$ gene therapy appears to be safe and feasible based on the 3-month interim follow-up. Definitive results of the trial will eventually bring a more profound insight on safety.

6.4 ETHICAL ASPECTS

Gene therapy has brought hope to treating multiple diseases that are incurable, the treatment methods available are insufficient, or have difficult adverse effects. In particular genetic diseases are an interesting target for gene therapy. Ethical issues have raised a lot of questions throughout the history of gene therapy. The idea of changing the functions of human body through manipulation of the genome may seem more extreme and irreversible than altering effects of enzymes or transmitters by pharmacological means. It should be noted however, that only gene therapy targeting somatic cells is permitted. Manipulation of germ cells could have effects reaching over generations and is currently prohibited [Kimmelman 2008]. As discussed earlier, malignancies and immunological reactions with a proven relation to gene therapy have been reported. The public attention is often focused on the negative outcomes and there is a risk that the positive achievements are left in the background. Due to the mutagenesis and inadequate innate immunity, gene therapy in children with SCID-X1 syndrome, seem to hold a risk of leukaemia of up to 50%. However, without the treatment the children would inevitably die at young age and would have to live the remaining life isolated from the normal environment. Gene therapy, despite the high risks, can provide cure and opportunity to live normal life for these children. Also the chance of surviving leukaemia is relatively high and does not seem to influence the effect of the treatment [Hacein-Bey-Abini et al. 2010]. In diseases such as SCID-X1 therapy can be justified even in small children unable to make the decision on their own. More ethical concerns might be raised in case of an adult patient, whose life does not depend on the treatment.

Inflammatory reactions are an important topic in gene therapy. The causes for the immunological reaction and death of the young OTCD patient have since been vigorously explored. Wilson and Allies, the principal investigators of the particular trial, have attempted to repeat the event in animal models, but have failed to see similar immune reactions. Also other patients recruited for the particular trial did not experience as severe responses. Although the cause remains unclear, the investigators suggest that genetic predisposition to enhanced immunity or immune memory to adenovirus could trigger such an excessive reaction [Wilson et al. 2009].

In cardiovascular gene therapy, new forms of treatment are targeted at elderly patients that have little hope of profiting from other treatment options. In addition, other experimental treatment options of refractory angina, such as transmyocardial laser revascularization have not shown sufficient evidence of efficacy [Manchanda et al. 2011]. Neurostimulation is an efficient option to some patients, but it is contraindicated in patients that are dependent on a pacemaker or defibrillator, have anatomical limitations to neurostimulation treatment or poor psychological co-operation [de Vries et al. 2007].

Cardiovascular stem cell therapy is under investigation, but has still many unsolved problems and a long way to go before becoming a treatment option [Madonna and De Caterina 2011; Sprengers et al. 2010; Tang et al. 2010]. In the absence of effective measures to treat patients with refractory angina, the potential adverse events of new treatment forms could more easily be accepted.

One of the challenges in the future is to continue developing vectors and delivery methods to further minimize known risk factors. On the other hand, all human beings are unique in their genetic background and unravelling the reasons why some patients react to the gene transfer differently than others could also provide important information. Whether the causes are related to genetic variation or differences in the acquired immune system, understanding these individual features could significantly contribute to the safety as well as efficiency of gene therapy.

7 Summary

The purpose of this doctoral work was to evaluate long-term safety of local VEGF-A gene transfer in patients with CAD and PAD. In addition, effects on exercise tolerance and incidence of major cardiovascular events and procedures were assessed. Furthermore, short-term safety aspects of intramyocardial AdVEGF-D$^{\Delta N \Delta C}$ via transseptal puncture were investigated.

Based on the results the following conclusions were made:

1. Local VEGF-A gene transfer did not increase mortality or the incidence of cancer or diabetes. Additionally, no significant increase in the incidence of diabetic retinopathy or nephropathy was detected.
2. Local VEGF-A gene transfer did not have any significant effects on exercise tolerance or cardiovascular events or procedures compared to placebo.
3. Intramyocardial AdVEGF-D$^{\Delta N \Delta C}$ gene transfer via transseptal puncture appears to be feasible and well tolerated.

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Appendices

Hyvä vastaanottaja,

Pyydämme Teitä ystävällisesti täyttämään tämän kyselylomakkeen YMPYRÖIMÄLLÄ oikea vaihtoehto.

1. Onko teillä tällä hetkellä mitään suorituskykyä rajoittavia oireita
   a. Ei
   b. Kyllä

2. Jos Teillä on suorituskykyä rajoittavia oireita, onko se oire
   a. Rintakipu
   b. Hengenahdistus
   c. Väsyminen
   d. Muu, mikä _____

3. Jos Teillä on suorituskykyä rajoittavia oireita, tulevatko oireet
   a. vasta kovassa rasituksessa (esimerkiksi ylämäkeen kävellessä tai portaita noustessa)
   b. jo kevyemmässä rasituksessa (esimerkiksi tasamaata kävellessä)
   c. levossa
   d. muulloin, milloin _____

4. Oletteko tällä hetkellä
   a. työssä
   b. sairaslomalla sepelvaltimotaudin takia
   c. sairaslomalla jonkin muun sairauden takia, minkä _____
   d. eläkkeellä sepelvaltimotaudin takia
   e. eläkkeellä jonkin muun sairauden takia, minkä _____
   f. eläkkeellä iän mukaisesti ilman sairautta

5. Onko Teillä ollut viimeisen geenihoitotutkimuskäynnin jälkeen sairaalahoitoa vaatinut sepelvaltimotautikohtaus (äkillinen sydäninfarkti tai rintakipuoireen äkillinen paheneminen)
   a. Ei
   b. Kyllä

6. Jos Teillä on ollut sairaalahoitoa vaatinut rintakipukohtaus,
   a. kuinka monta kertaa _____
   b. milloin se/ne oli(vat) _____
   c. missä sairaalassa olitte hoidossa _____

7. Jos olitte sairaalahoidossa sepelvaltimokohtauksen takia, tehtiinkö teille sepelvaltimoiden varjoainekuvaus
   a. Ei
   b. Kyllä

8. Jos olitte sairaalahoidossa sepelvaltimokohtauksen takia, tehtiinkö teille sepelvaltimoiden pallolaajennus ja stenttaus
   a. Ei
   b. Kyllä

9. Jos olitte sairaalahoidossa sepelvaltimokohtauksen takia, tehtiinkö Teille sepelvaltimoiden ohitusleikkaus
   a. Ei
   b. Kyllä

10. Onko Teille geenihoitotutkimukseen osallistumisenne jälkeen jouduttu tekemään tutkimuksia sydämen rytmihäiriöiden takia
   a. Ei
   b. Kyllä

11. Jos Teille on jouduttu tekemään rytmihäiriötutkimuksia, milloin ja missä sairaalassa ne on tehty _____

_____

12. Onko Teille jouduttu asentamaan sydämen tahdistinta geenihoitotutkimukseen osallistumisenne jälkeen
   a. Ei
   b. Kyllä

13. Oletteko joutuneet sairaalahoitoon jonkin muun kuin sepelvaltimotaudin takia geenihoitotutkimukseen osallistumisen jälkeen
   a. Ei
   b. Kyllä

14. Jos olette joutuneet sairaalahoitoon muun kuin sepelvaltimotaudin takia, minkä takia ja milloin ja missä sairaalassa olitte hoidossa

_____

_____

_____

15. Onko Teillä todettu syöpäsairautta geenihoitotutkimukseen osallistumisen jälkeen
   a. Ei
   b. Kyllä, mikä _____

16. Jos Teillä on todettu syöpäsairaus geenihoitotutkimukseen osallistumisen jälkeen, missä sairaalassa sairauttanne on hoidettu ja milloin

_____

_____

17. Onko Teillä todettu sokeritautia geenihoitotutkimukseen osallistumisen jälkeen
   a. Ei
   b. Kyllä

18. Jos Teillä on todettu diabetes geenihoitotutkimukseen osallistumisen jälkeen, onko siihen liittyen todettu munuaisten vajaatoimintaa
   a. Ei
   b. Kyllä

19. Jos Teillä on todettu diabetes geenihoitotutkimukseen osallistumisen jälkeen, onko siihen liittyen todettu silmänpohjan muutoksia
    a. Ei
    b. Kyllä

20. Onko Teillä todettu aivoverenkiertohäiriöitä geenihoitotutkimukseen osallistumisen jälkeen
    a. Ei
    b. Kyllä

21. Jos Teillä on todettu aivoverenkiertohäiriöitä geenihoitotutkimukseen osallistumisen jälkeen, missä sairaalassa sairauttanne on hoidettu ja milloin

    _____

    _____

22. Onko Teillä todettu jokin muu pitkäaikaissairaus geenihoitotutkimukseen osallistumisen jälkeen
    a. Ei
    b. Kyllä, mikä _____

23. Jos Teillä on todettu jokin muu pitkäaikaissairaus geenihoitotutkimukseen osallistumisen jälkeen, missä sairaalassa sairauttanne on hoidettu ja milloin

    _____

    _____

24. Miten koitte geenihoitotutkimukseen osallistumisen vaikuttaneen elämänlaatuunne
    a. Ei mitenkään
    b. Paransi vointia
    c. Huononsi vointia
    d. En osaa sanoa

Muuta kommentoitavaa _____

_____

_____

LÄMMIN KIITOS VAIVANNÄÖSTÄNNE. HYVÄÄ JATKOA.

Hyvä vastaanottaja,
pyydämme teitä ystävällisesti YMPYRÖIMÄÄN oikean, Teitä koskevan, vaihtoehdon.

Oletteko tällä hetkellä työelämässä?

a) Kyllä
b) Eläkkeellä sairauden takia, minkä?_____
c) Vanhuuseläkkeellä
d) Sairauslomalla, miksi?_____

Onko Teillä toimenpiteen jälkeen esiintynyt alaraajoissa kipuoireita, ns. katkokävelyä?

a) Kyllä
b) Ei

Jos kyllä, mikä seuraavista kuvaa parhaiten suorituskykyänne tällä hetkellä?

a) Kävelymatka yli 100m
b) Kävelymatka alle 100m
c) Lepokipua alaraajoissa
d) Haavaumia tai kuolioita alaraajoissa

Onko Teillä rasituksessa esiintyvää rintakipua tai hengenahdistusta?

a) Ei
b) Kyllä

Jos kyllä, mikä seuraavista kuvaa rintakivun/hengenahdistuksen osalta suorituskykyänne parhaiten?

a) Oireita vain kovassa rasituksessa
b) Oireita tasamaata kävellessä
c) Oireita levossa

Jos Teillä esiintyy sekä rintakipua/hengenahdistusta sekä alaraaja-oireita, onko liikkumista ensimmäiseksi rajoittava oire:

a) Hengenahdistus/rintakipu
b) Alaraajakipu

Onko Teille tehty alaraajojen varjoainetutkimusta seuranta-aikana?

a) Ei
b) Kyllä

Jos kyllä, kumpaan alaraajaan varjoainekuvaus tehtiin?

a) Oikeaan, montako kertaa?_____
b) Vasempaan, montako kertaa?_____
c) Molempiin, montako kertaa?_____

Jos kyllä, tehtiinkö pallolaajennus?

a) Ei
b) Kyllä

Onko Teille tehty alaraajojen ohitusleikkausta seuranta-aikana?

a) Ei
b) Kyllä, milloin?_____

Onko Teille tehty alraaja-amputaatiota seuranta-aikana?

a) Ei
b) Kyllä, milloin?_____

Onko Teillä todettu syöpää seuranta-aikana?

a) Ei
b) Kyllä, mikä?_____
_____

Onko Teillä todettu diabetesta seuranta-aikana?

a) Ei
b) Kyllä

Mikäli Teillä on todettu diabetes, onko todettu jokin seuraavista liitännäissairauksista?

a) Munaisten vajaatoiminta
b) Verkkokalvon muutokset

Onko Teillä todettu jotain muuta pitkäaikaissairautta seuranta-aikana?

a) Ei
b) Kyllä, mikä?_____
_____
_____
_____
_____

Kommentteja: _____
_____
_____
_____
_____

Kiitos vastauksistanne!

Kirsi Muona

Safety of VEGF Gene Therapy in Cardiovascular Diseases

VEGF mediated gene therapy is a promising treatment method for patients with advanced cardiovascular diseases. However, it involves theoretical short- and long-term risk factors that require careful assessement. The aim of this thesis was to investigate the short-term safety aspects of VEGF-$D^{\Delta N \Delta C}$ gene therapy as well as long-term effects and safety of VEGF-A gene therapy in patients with coronary and peripheral artery diseases.

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