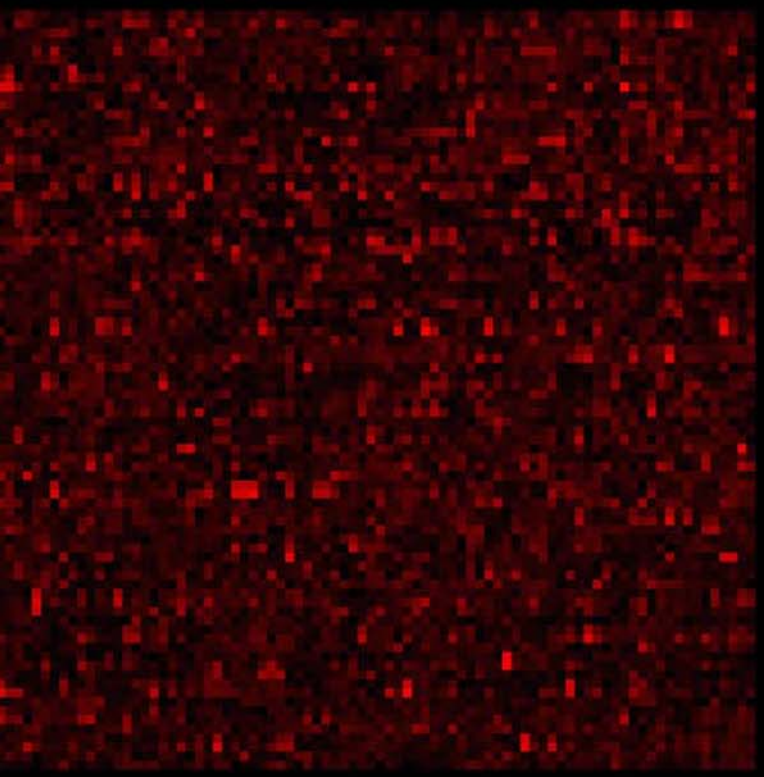
Thesis for doctoral degree (Ph.D.)
Stockholm 2006

Myocardial Gene Therapy and Gene Expression in Angina Pectoris



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Published by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden
Printed by Universitetsservice US-AB

ISBN 91-7140-648-4

ABSTRACT

Background
Angiogenesis does not fully counteract myocardial ischemia in stable angina pectoris. Refractory angina pectoris, with remaining symptoms despite medication and no possibility for bypass surgery or angioplasty, is rather common. Angiogenic gene therapy is a novel treatment strategy for these patients.

Methods and results
In study I, six patients with refractory angina received intramyocardial injections of 0.25-1 mg plasmid encoding Vascular Endothelial Growth Factor (phVEGF-A_{165}) via thoracotomy. The peak systolic velocity improved in all six patients but perioperative myocardial infarction occurred in two patients.

Study II was a double-blind randomised controlled trial of the same plasmid or placebo plasmid (0.5 mg), delivered via a percutaneous catheter system in 80 patients with refractory angina. Reversible perfusion defects and wall motion improved in the phVEGF-A_{165}-treated area compared to placebo. Nitroglycerin use tended to decrease with active treatment while symptom class and exercise capacity showed no effect beyond placebo. Five catheter-related adverse events occurred but no adverse effects were related to the plasmid.

In study III, the prognosis of refractory angina was assessed in all 225 patients screened for study II. The mortality was 10.6% at three years. The baseline screening angiogram found revascularisation options in 10% of patients, although previous examinations had ruled out such possibilities. After twelve months, 36% of the trial patients had improved by at least two symptom classes and 37% had increased their exercise time by at least 60 seconds, with no difference between placebo and active groups.

In study IV-V, the gene expression pattern in a reversibly ischemic myocardial area was compared to a normal area in eight patients with stable angina pectoris. Real-time polymerase chain reaction showed increased expression of ANP and BNP but not of VEGF and VEGF receptor 1 and 2 in reversibly ischemic myocardium. In microarray measurements, 15 additional known angiogenesis stimulators lacked differential expression. Instead, we found increased expression of several other genes with potential angiogenic, angiogenesis inhibiting, anti-apoptotic and muscle-related function but with yet unknown role in the myocardium.

Conclusions
Intramyocardial phVEGF-A_{165} is safe and increases myocardial perfusion in patients with stable angina pectoris. The effect on symptoms should be tested in a larger trial. Patients with refractory angina pectoris have a rather low mortality and symptomatic improvement is common. Overexpressing VEGF (or other angiogenic factors) seems a rational strategy, as most angiogenesis stimulators not are overexpressed in ischemic myocardium in stable angina. The ischemia-related overexpression of ANP, BNP and other genes with a probable anti-angiogenic function might be a limiting factor in angiogenesis.

Keywords
angina pectoris, gene therapy, vascular endothelial growth factor, plasmid, prognosis, collaterals, angiogenesis, gene expression, microarray, natriuretic peptides.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Sylvén C, Sarkar N, **Rück A**, Drvota V, Hassan SY, Lind B, Nygren A, Källner G, Blomberg P, van der Linden J, Lindblom D, Brodin LA, Islam KB. **Myocardial Doppler tissue velocity improves following myocardial gene therapy with VEGF-A165 plasmid in patients with inoperable angina pectoris.** *Coron Artery Dis. 2001;12(3):239-43*

II. Kastrup J, Jørgensen E, **Rück A**, Tagil K, Glogar D, Ruzyllo W, Bøtker HE, Dudek D, Drvota V, Hesse B, Thuesen L, Blomberg P, Gyöngyösi M, Sylvén C. **Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial.** *J Am Coll Cardiol. 2005;45(7):982-8*

III. **Rück A**, Drvota V, Kastrup J, Dudek D, Bøtker HE, Ruzyllo W, Gyöngyösi M, Glogar D, Sylvén C. **Favourable prognosis in refractory angina pectoris A three-year follow-up of 225 patients.** *Manuscript, submitted.*

IV. **Rück A,** Gustafsson T, Norrbom J, Nowak J, Källner G, Söderberg M, Sylvén C, Drvota V. **ANP and BNP but not VEGF are regionally overexpressed in ischemic human myocardium.** *Biochem Biophys Res Commun. 2004;322(1):287-91*

V. **Rück A**, Gustafsson T, Norrbom J, Nowak J, Källner G, Söderberg M, Sylvén C, Drvota V. **The gene expression profile of stable angina pectoris in human myocardium.** *Manuscript.*

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1 ABBREVIATIONS

ANP	Atrial Natriuretic Peptide
BNP	Brain Natriuretic Peptide
ECG	Electrocardiogram
FGF	Fibroblast Growth Factor
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HIF-1α	Hypoxia Inducible Factor 1α
LAD	Left Anterior Descending coronary artery
LIMA	Left Internal Mammary Artery
MCP-1	Monocyte Chemoattractant Protein 1
MRI	Magnetic Resonance Imaging
NOGA	trademark of an electromechanical mapping system
PCI	Percutaneous Coronary Intervention
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PET	Positron Emission Tomography
PlGF	Placental Growth Factor
phVEGF-A_{165}	plasmid encoding human Vascular Endothelial Growth Factor-A 165
ROI	Region Of Interest
SPECT	Single Photon Emission Computed Tomography
TGFß	Transforming Growth Factor beta
VEGF	Vascular Endothelial Growth Factor

2 Mr. A – an example of limitations of current therapies for stable angina pectoris

Mr. A. is 63 years of age. His ischemic heart disease started with a myocardial infarction ten years ago, after which he had angina pectoris. In the following year he underwent bypass surgery and the chest pain and shortness of breath subsided. Apart form moderate hyperlipidemia, he has no other diseases.

Now he is admitted to the hospital with chest pain and a non-ST-elevation myocardial infarction. The ECG shows lateral ST-depression. The systolic left ventricular function is slightly depressed with an ejection fraction of 40%.

He is stabilized with standard pharmacologic treatment.

A new coronary angiogram (next page) shows an occlusion of the LAD, severe diffuse stenosis of the circumflex and subtotal occlusion of the right coronary artery. The LIMA graft to the LAD is open as well as a vein graft to the right coronary. Vein grafts to the circumflex and to a diagonal branch are occluded.

It is suspected that the myocardial ischemia is located in the circumflex territory. This vessel is not considered suitable for new bypass surgery or PCI. Mr. A. is discharged from the hospital with optimised medication.

A SPECT perfusion scan (below) showed a reversible perfusion defect in the lateral wall, corresponding to the circumflex stenosis, and a permanent perfusion defect in the inferior wall, corresponding to the myocardial infarction ten years ago.

A few weeks later, Mr. A. comes to the policlinic. He suffers from frequent attacks of chest pain even at a low level of exercise.

Will gene therapy help him? Will his symptoms improve? Is he at high risk of dying? Which genes are active in his ischemic area? Why does his recurrent ischemia not cure him from angina pectoris by inducing collateral arteries?

These are the questions this thesis tries to answer.

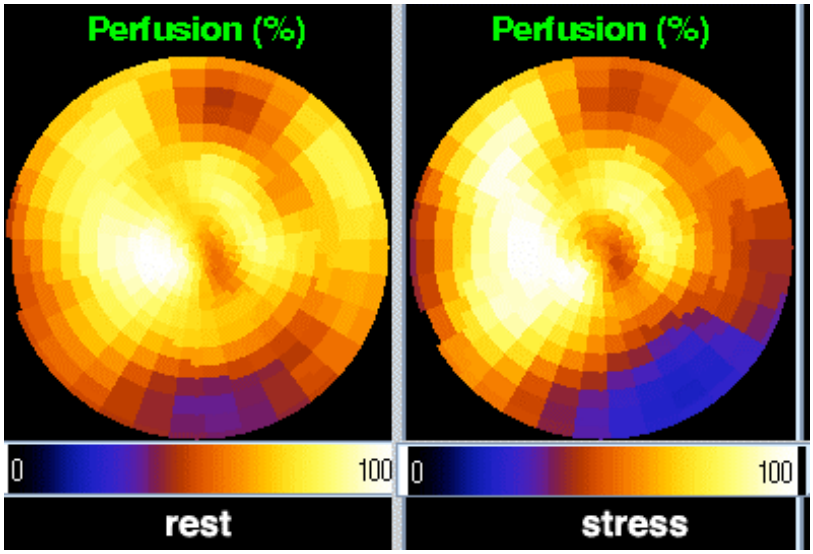

Fig. 1. SPECT scan, polar plot. Apex is in the center of each circle, the circumflex territory is seen to the right in each circle.

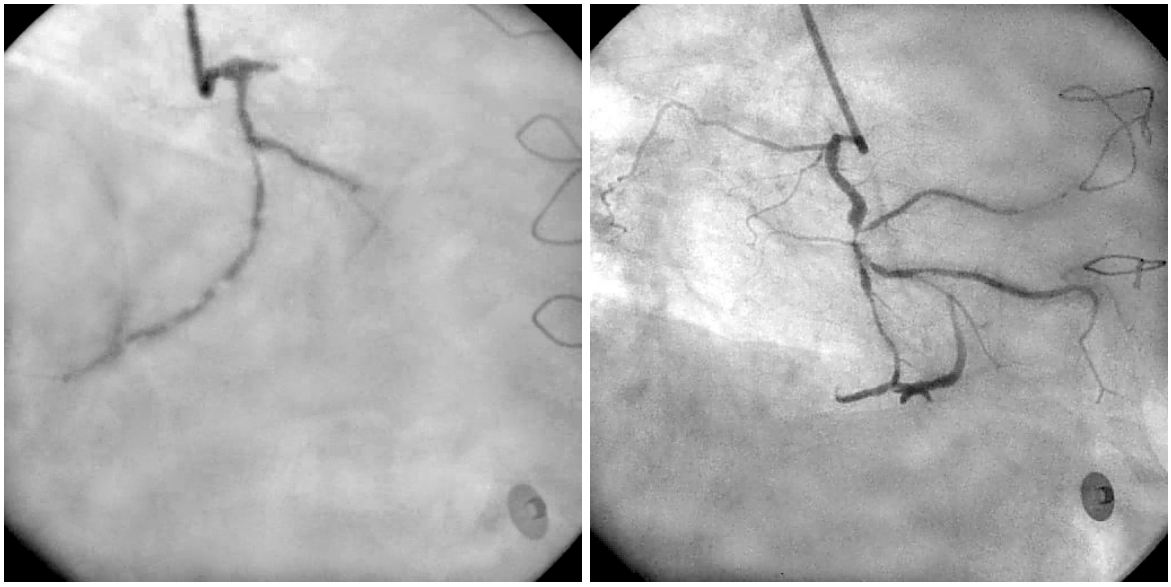

Fig. 2. Coronary angiography. Native coronary arteries: occluded LAD and severe stenosis of the circumflex (left), subtotal occlusion of the right coronary (right).

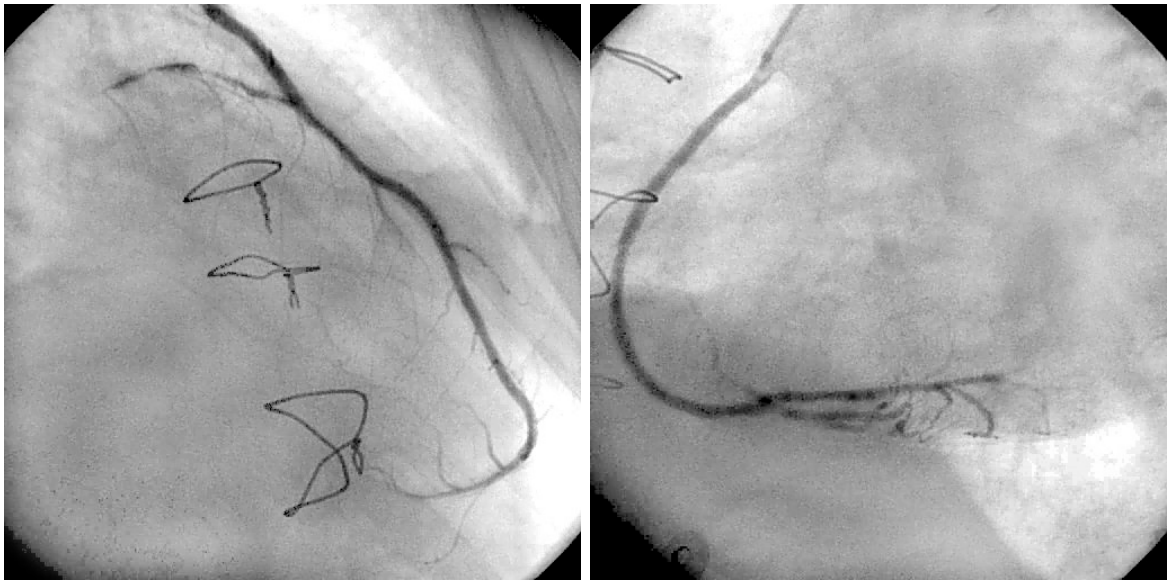

Fig. 3. Coronary angiography. Grafts: open LIMA to LAD (left), open vein to right coronary (right). Vein grafts to the circumflex and to a diagonal branch were occluded (not shown).

3 INTRODUCTION

3.1 CORONARY ARTERY DISEASE – A MAJOR HEALTH PROBLEM

Coronary heart disease is the most common cause of death both worldwide and in Sweden [1]. While most of the mortality is caused by acute myocardial infarction and the associated arrhythmias and heart failure, a large burden of morbidity is caused by chronic ischemic heart disease [2]. Patients with chronic angina pectoris have a decreased quality of life and may have chest pain and shortness of breath even at minimal exertion [3].
Most patients with stable angina pectoris can be successfully treated with a combination of medication and revascularisation with either bypass surgery or percutaneous coronary intervention. However, some patients remain symptomatic despite optimal medication and are not suitable for revascularisation. It has been calculated that 2-5% of patients referred for coronary angiography because of stable angina cannot be revascularised [4]. Many of these patients have diffuse and distal atherosclerosis, which makes PCI difficult and bypass surgery unlikely to help, as the recipient vessel is of small calibre and poor quality. These patients often have had a prior bypass operation, after which vein grafts have degenerated but the arterial graft remains open. A second bypass procedure has a higher procedural risk than the first one, especially with higher age and concominant disease such as renal dysfunction and diabetes. There is also a risk of damaging the functional arterial graft.
These patients have been called refractory angina pectoris, a term that is defined in a task force report from the European Society of Cardiology [5].

Refractory Angina Pectoris

"A chronic condition characterized by the presence of angina pectoris caused by coronary insufficiency in the presence of coronary artery disease which cannot be controlled by a combination of medical therapy, angioplasty and coronary bypass surgery. The presence of reversible myocardial ischaemia should be clinically established to be the cause of the symptoms. Chronic is defined as a duration of more than 3 months."

Ominous sounding terms like "end-stage coronary disease" [6, 7] and "no-option" [8-10] have been used for these patients, suggesting a sinister prognosis, although there is a lack of data to support that common view.

3.1.1 New therapies

Several types of therapies have been investigated for patients with refractory angina pectoris.
A *Spinal cord stimulator (SCS)* is an implanted device which has been shown to have similar symptomatic effect as bypass surgery in patients with no prognostic benefit of bypass surgery or increased surgical risk [11-13]. Decreased myocardial oxygen

demand and possibly blood flow redistribution are the suggested mechanisms of action. Due to the paresthesia caused by the stimulation, it has not been possible to conduct a double-blind randomised trial. In Sweden the highest number of SCS devices are implanted in Gothenburg.

Enhanced external counterpulsation (EECP) inflates pressure cuffs around the patients' legs and pelvis during diastole, which induces diastolic pressure augmentation in a similar manner to invasive aortic balloon pumping. A therapy session lasts for one hour, and typically the patient receives 35 sessions during seven weeks. Decreased symptoms have been documented in registry studies for over 2 years after therapy [14]. The MUST-EECP study is the only randomised trial [15]. Patients were randomised to either normal pressure pumping (300 mmHg) or a low pressure of 75 mmHg. The only significant difference in the intention-to-treat analysis after seven weeks was time to ST-depression on the treadmill test, while nitroglycerin use and angina counts only showed trends to improvement. The mechanism of action is unclear even if it is hypothesized that the increased diastolic pressure induces collateral growth in the heart [16, 17]. On the other hand a recent study found no improvement in myocardial perfusion on SPECT [18]. EECP is now available in several hospitals in Sweden.

Laser revascularization was performed at many centers in the nineties but has now largely been abandoned. During this procedure, which is possible via a percutaneous catheter system or via thoracotomy, a number of transmyocardial channels are created with a laser. Several trials have reported symptomatic improvement in randomised non-blinded trials with continued medical treatment as control [19, 20]. A double-blind randomised trial, DIRECT, with a percutaneous system, showed a substantial improvement in the placebo group, with no difference to active treatment [21]. Laser revascularisation is not performed in Sweden any more.

Physical exercise might be advantageous but has not been studied in refractory angina pectoris per se. Exercise training has recently shown a similar symptomatic effect and better exercise tolerance after one year compared to PCI in a randomised open trial [22]. Perfusion on SPECT also improved after exercise training [23]. Exercise stimulates several signal transduction pathways leading to antiapoptotic effects and increased nitric oxide availability [24].

In the late nineties, animal data [25] and small human trials [26] indicated that enhanced blood vessel growth, *angiogenesis*, might dramatically diminish myocardial ischemia and its associated symptoms. In this era the work on this thesis was initiated.

3.2 MECHANISMS OF ANGIOGENESIS AND ARTERIOGENESIS

3.2.1 Angiogenesis – good and bad

It has long been known that postnatal blood vessel growth, angiogenesis, occurs in some specific situations. Relevant human examples are the female reproductive tract during the menstrual cycle and pregnancy and also wound healing. Apart from this physiological role, angiogenesis is also important in the pathogenesis of tumours, inflammatory disorders and diabetic retinopathy. On the other hand, insufficient angiogenesis and blood supply is found not only in ischemic heart disease but also in peripheral arterial atherosclerosis, diabetes, preeclampsia and Crohns disease [27]

Angiogenesis-inhibiting drugs are already in clinical use for the treatment of tumours, while the therapeutic stimulation of angiogenesis for ischemic diseases still is under intense scientific development.

3.2.2 Angiogenesis vs. arteriogenesis

Angiogenesis is often used as a general term for postnatal blood vessel growth. In recent years the term arteriogenesis has been used for the enlargement of pre-existing vessels (such as coronary collaterals) [28, 29], while angiogenesis in its more restricted sense refers to capillary growth. The mechanisms of arteriogenesis and angiogenesis are different, although many stimuli elicit both responses [30]. Both the physical force of blood flow itself, growth factors and progenitor cells take part in this intricate process.
The flow capacity of a vessel increases with the fourth power of its radius, which shows the high functional impact of a rather small enlargement of the collateral vessels. It is also obvious that even a huge amount of capillaries cannot replace the flow capacity of a larger conductance vessel. An increased number of capillaries, induced by hypoxia, may on the other hand increase the flow to the myocardium by lowering the resistance. This increase in flow induces enlargement of the supplying collateral artery by increased fluid shear stress.
Thus angiogenesis and arteriogenesis are dependent on each other, as higher collateral flow requires an adequate capillary network in the myocardium, and newly grown capillaries depend on increased blood flow in the supplying artery.
In coronary heart disease, collateral growth is needed upstream and adjacent to the ischemic region, while capillary growth within the ischemic region increases the nourishing of the ischemic or hibernating myocardium [31].

3.2.2.1 Arteriogenesis (collateral growth)

Arteriogenesis is stimulated by increased flow shear stress but only to a limited degree by ischemia [28, 32]. The translation of the mechanical force to the cellular level is not completely understood. Adhesion molecules such as VCAM and ICAM and the monocyte chemoattractant MCP-1 are important, as well as monocytes and endothelial progenitor cells. Growth factors such as VEGF, FGF, PlGF, TGFß and also the stem-cell releasing factors G-CSF and GM-CSF have been shown to augment arteriogenesis and angiogenesis. The growth and maintenance of the artery size does not only involve endothelial cells but also supporting smooth muscle cells and pericytes, which is influenced by PDGF. The surrounding extracellular matrix is also remodelled to accommodate the growing artery. This remodelling is accomplished by proteinases such as plasminogen activators (PAI-1) and matrix metalloproteinases [27, 33]. Both angiogenic activators (VEGF, VEGF, TGFß) and inhibitors (trombospondin, endostatin) are liberated from their matrix-bound state during the remodelling process.

3.2.2.2 Angiogenesis (capillary growth) and ischemia

Angiogenesis is largely regulated by tissue hypoxia and ischemia. Hypoxia directly inhibits the hydroxylation of the transcription factor HIF-1α, dramatically increasing its cellular levels within minutes. HIFα induces the transcription of VEGF, VEGF receptors 1 and 2, nitric oxide synthases and PAI-1 [34]. Indirectly, FGFs, Angiopoietin-2, Tie-2, MCP-1 and PDGF are induced.
While acute ischemia both in animal models and in clinical myocardial infarction induces these dramatic gene expression changes, it is not clear if the same changes

occur for a prolonged time in stable angina pectoris. Ironically, we do not know the myocardial gene expression pattern in the very patients we treat with gene therapy in our trials. Animal data suggests that repetitive short myocardial ischemia causes an initial increase of VEGF and other growth factors, but this response is blunted with time [35].

3.2.2.3 VEGF and its receptors

VEGF is the most studied angiogenic factor. The VEGF family consists of at least six members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placental growth factor (PlGF)), each coded by a separate gene [36]. VEGF-A is thought to be most important variety in angiogenesis and is often referred to as just VEGF. VEGF-C is important in lymphangiogenesis.

VEGF-A exists in six isoforms as the result of alternative splicing. All isoforms contain a secretory signal, enabling VEGF to have paracrine effects on surrounding cells. The longer the isoform, the higher its affinity to extracellular proteoglycans. VEGF-A_{165} is intermediate in length, with balanced properties between extracellular retention and diffusibility, and optimal angiogenic potency.

Hypoxia induces increased expression of VEGF through HIF1-α but does also increase the half-life of the already expressed VEGF mRNA [34]. Inflammatory reactions and many cytokines (TGFß, Interleukin-6, Insulin like growth factor-1, PDGF, FGF) also induce VEGF expression.

The actions of VEGF are mediated by three tyrosine kinase receptors: VEGF-R1 (a.k.a. flt-1), VEGF-R2 (a.k.a. KDR or flk-1) and VEGF-R3. The latter mainly interacts with VEGF-C in lymphangiogenesis. VEGF-R1, which also exists in a soluble circulating form, does not have strong angiogenic effects, but might act as a decoy receptor and regulate VEGF effect. The chemotaxis of monocytes seems to be mediated by VEGF-R1, and it might also cause tissue-specific release of other growth factors.

VEGF-R2 is the key mediator of the angiogenic effects and induces the phosphorylation of several proteins in endothelial cells.

VEGF-A binds to both VEGF-R1 and R2. In addition, the neurolipin receptors (NRP1 and NRP2) act as co-receptors and enhance the response of VEGF-R2 to VEGF-A.

3.2.2.4 Angiogenesis inhibitors

The presence of numerous interrelated stimulators of angiogenesis is further complicated by the presence of inhibitors of angiogenesis. Examples are thrombospondin-1, angiopoietin-2 (in the absence of VEGF), soluble circulating VEGF receptors, cleavage products of matrix components (arresten, vastatin, endostatin) and cleavage products of plasma proteins (angiostatin, serpins) [27]. Signalling molecules such as the atrial and brain natriuretic factors (ANP and BNP) [37, 38] and TGFß [39-41] also have antiangiogenic effects apart from their role in heart failure.

In the clinical setting, diabetes [42], hypercholesteremia [43-46] and higher age [47, 48] may also impair the angiogenic response. Furthermore common cardiovascular medication such as ACE-inhibitors may inhibit angiogenesis [49].

3.2.3 Collaterals in coronary artery disease

Although the presence and importance of coronary collaterals in diminishing myocardial ischemia has been appreciated for a long time, it is a new finding that a considerable collateral circulation exists also in humans without coronary stenosis [50].

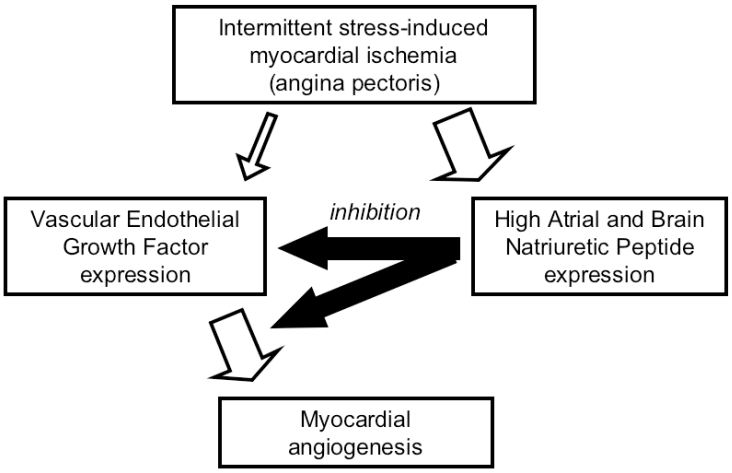

Fig. 4.
ANP and BNP counteract VEGF. Unfilled arrows show stimulation, filled arrows inhibition.

The extent of collateral flow is highly variable between individuals, both with and without coronary stenosis. There is a moderate correlation between stenosis severity and collateral flow [51].
Collateral flow in the heart can be assessed in vivo by several methods [52]. The most common is Rentrop grading during coronary angiography [53]. In the most used form, the spontaneous filling of the collateral receiving artery is graded from 0 to 3. The method originally described by Rentrop uses the same score during balloon occlusion of the collateral receiving artery, measuring the recruitable collateral flow. A less common method is washout collateralometry, where the number of heart beats is counted before the contrast is washed out, during balloon occlusion [54]. These angiographic visual methods have a rather low sensitivity and are probably too crude for quantification of collateral flow in studies. They are also prone to error by variations in contrast concentration, heart rate and image quality.

3.2.3.1 Collateral flow index

A more precise measurement is possible by measuring the intracoronary pressure or flow velocity in the collateral donor artery, distal to a brief balloon occlusion [55]. Pressure measurements are independent of the position of the measuring guidewire in the lumen and therefore more reproducible. The pressure is measured after one minute of balloon occlusion, which provides an ischemic stimulus to dilate the collateral vessels. To obtain the collateral flow index (CFI), the distal pressure is divided by the aortic pressure, after subtraction of the venous pressure. A CFI over 0.25 is interpreted as sufficient collateral flow to prevent ischemia during brief vessel occlusion [52].

3.2.3.2 Indirect measurements of collateral flow – perfusion imaging

In the case where the supplying coronary artery is totally occluded, the blood flow to that myocardial region must be via collaterals, and measurement of perfusion with any imaging method will be an estimate of collateral flow. If the supplying artery has a stenosis, a change in perfusion would reflect changed collateral flow, if the stenosis severity is constant.
The most common perfusion imaging method is SPECT, which has been used in many angiogenesis trials. However it has been shown that SPECT perfusion defects have a considerable variation over time in individual patients, even if the mean perfusion

defect size in a group stays rather constant [56]. In other words, the standard deviation might be high. The use of SPECT as an endpoint in small unblinded studies might therefore be inappropriate. Even in angiographic three-vessel disease, 18% of scans will not show reversible perfusion defects [57, 58]. Advantages with SPECT include its wide availability and the large experience in image analysis [59].
Cardiac stress MRI is a newer method for perfusion imaging which might be advantageous as it has higher spatial resolution [60].
Unlike SPECT and MRI, which measure relative perfusion, PET [61] and contrast stress echo [62] are able to quantify absolute perfusion. However these methods have not yet reached a widespread use.
The measurement of regional wall motion may also be seen as an indirect measurement of perfusion and collateral flow. Regional wall motion can be measured with tissue Doppler or visual scoring (echocardiography), the centerline method (left ventricular angiography) [63], wall motion (MRI) [60] or Linear local shortening (LLS) on NOGA imaging [64, 65]. Stress echocardiography with tissue velocity imaging has been shown to be sensitive to myocardial ischemia. It is a more quantitative measurement than the conventional visual scoring system, and might therefore be more suitable to detect changes over time in the same segment and patient [66]. Recently strain rate measurement has evolved as a more sensitive measurement of ischemia [67]. Strain rate, unlike systolic velocity, is not affected by forces in the adjacent myocardium.

3.2.4 Premature plateau of collateral growth

Collateral growth seems to start within a week and continue for a few months after the occlusion of a coronary artery in humans [68]. The end result does rarely if ever reach the same high flow capacity and low resistance as the compromised artery it should replace [28]. Thus, this compensatory process stops at an incomplete stage of adaptation. The flow capacity of the collaterals is usually enough for the demand of the myocardium at rest and light exercise. But as the coronary flow cannot increase further, the patients usually suffer from chest pain and shortness of breath during exercise.
It is not known why collateral growth stops at this premature plateau. It might be that the early decrease of flow shear stress, the main driving force in arteriogenesis, during collateral growth is the reason. Other reasons could be that VEGF and other angiogenic substances not are induced any more after several weeks of intermittent moderate myocardial ischemia [35]. Inhibitory factors may also be the limiting factor.

3.3 THERAPEUTICALLY INDUCED ANGIOGENESIS

The idea to augment the perfusion of ischemic myocardium by enhancing collateral circulation is not new. Procedures like asbestos powdering of the pericardial sac, tacking omentum to the heart [69] or implanting the internal mammary artery directly into the myocardium without anastomosis to a recipient vessel (Vineberg) have been used [70]. Although these techniques were abandoned after aortocoronary bypass surgery was developed, there is reason to believe they had a therapeutic effect [71].

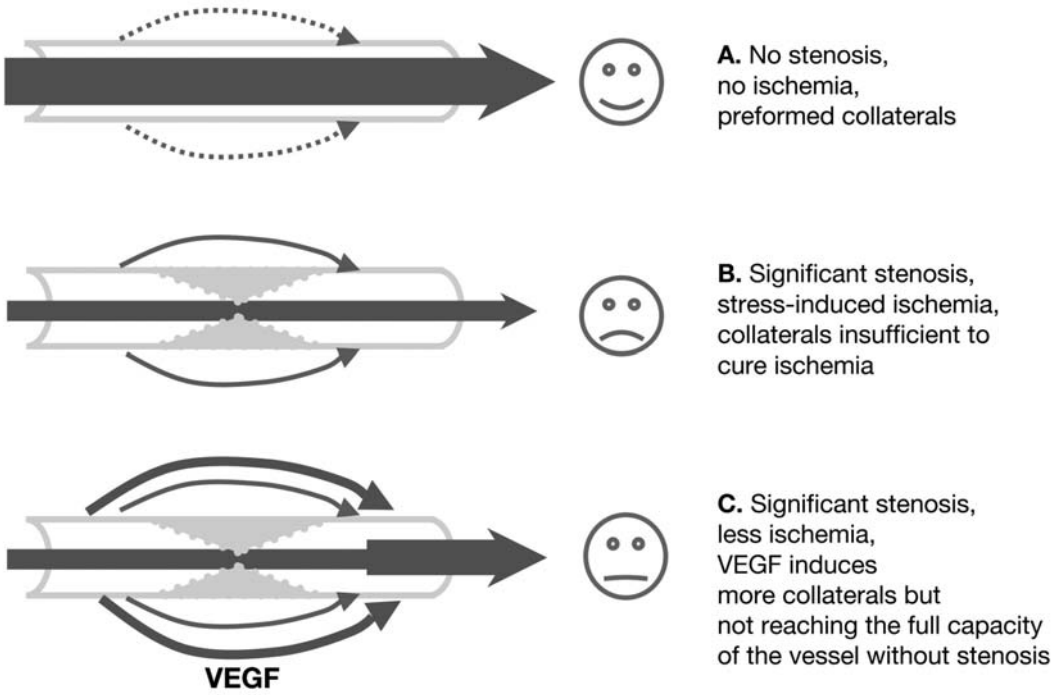

Fig. 5. Principles of collaterals and therapeutic angiogenesis.
The faces show the ischemic situation of the myocardium supplied by the artery.

As the molecular mechanisms of angiogenesis and arteriogenesis were unravelled [72], it became clear that there was a therapeutic potential in enhancing this naturally occurring process [26].

Gene therapy has been used as a "slow-release" preparation to overcome side effects of proteins and achieve the prolonged protein level required for angiogenesis. Gene transfer can be achieved with plasmids (via passive diffusion) or with viral vectors, which bind to cellular receptors. Plasmids are relatively easy to produce and have few side-effects. Viral vectors transfect a much higher proportion of cells, but at the price of an inflammatory reaction [73].

In ischemic heart disease, enhanced angiogenesis could be applied as an alternative to traditional revascularisation strategies in order to diminish ischemia. This could be used when traditional procedures not can be applied (refractory angina), but also earlier on in the disease process, like in the hibernating area surrounding an acute myocardial infarction. It is also conceivable to prophylactically augment the collateral circulation in order to prevent the damage of a possible future acute coronary occlusion.

As mentioned, angiogenesis involves multiple steps and growth factors. Promising results in animal studies have been shown with several genes including VEGF [25, 74-79], FGF [80, 81], PDGF [82-84], MCP-1 [85] and HIF1-α [86, 87].

Clinical trials of angiogenic gene therapy with FGF [88, 89] and VEGF [90, 91] in coronary artery disease have until now not shown any symptomatic benefit above placebo in randomised trials, even if the effect in smaller unblinded trials was substantial [92]. There have however been encouraging results and trials on various agents are ongoing.

4 GENERAL AIMS

a. To assess the safety and therapeutic efficacy of intramyocardial injections of plasmid encoding VEGF-A_{165} in patients with refractory angina pectoris (studies I-II).

b. To investigate the prognosis (mortality, new revascularization options and symptomatic improvement) in patients with a clinical diagnosis of refractory angina pectoris (study III).

c. To investigate the gene expression pattern in a reversibly ischemic and a normal area of the left ventricle in patients with stable angina pectoris (studies IV-V).

5 METHODS

Permission for the studies was obtained from the local Ethics Committee. Permission for studies I and II was also obtained from the Swedish Medical Products Agency. All patients gave their informed and written consent.

5.1 STUDY I

Patients
Inclusion criteria were Canadian Cardiovascular Society (CCS) functional class III-IV angina pectoris refractory to optimal medical treatment, not eligible for invasive treatment. Further requirements were at least one patent major vessel related to the anterolateral part of the left ventricle and viable areas of anterolateral left ventricular myocardium with major reversible ischaemia involving at least 10% of the left ventricle and detectable with adenosine single-photon emission computerized tomography (SPECT). Patients with an ejection fraction < 20%, unstable angina pectoris during the last 3 months, cancer, chronic inflammatory disease or diabetic retinopathy were excluded.

SPECT, coronary angiography and stress echocardiography
At baseline and at follow-up 2 months after treatment, adenosine stress SPECT, coronary angiography and dobutamine stress echocardiography with tissue velocity imaging (TVI) were performed. Both SPECT and coronary angiography were evaluated with the before and after treatment examinations in random order, thus blinding the observers.
For SPECT, perfusion at rest, stress and reversible perfusion defects (stress-rest) were evaluated and categorized as impaired (-2), slightly impaired (-1), unchanged (0), slightly improved (+1) or improved (+2).

Operation and plasmid administration
Under general anaesthesia and with cardiac monitoring by transoesophageal echocardiography, a left lateral thoracotomy of about 10 cm was made in the fifth inter-costal space. Under direct visualization, 0.25 (4 patients) or 1.0 (2 patients) mg phVEGF-A_{165} in 8ml saline divided in four 2ml aliquots was injected into the previously localized ischemic area.

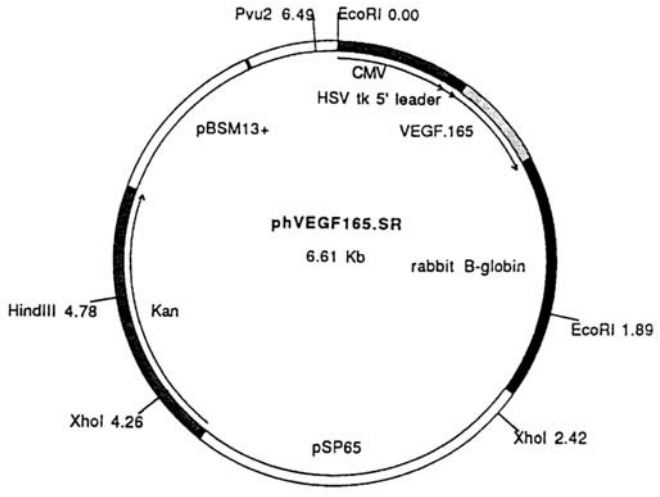

Fig. 6. Plasmid encoding VEGF-A165, schematic. Expression is driven by a Cytomegalovirus (CMV) promotor.

Plasmid preparation and VEGF-A assay
The plasmid phVEGF-A_{165} was originally a generous gift from the late Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, USA, and was produced at the Gene Therapy Center (Huddinge, Sweden) according to Good Manufacturing Practice (GMP) standards. Production, purity and sterility were controlled as specified by the Swedish Medical Products Agency. The plasmid contained a cytomegalovirus promotor/enhancer to drive VEGF-A_{165} expression.
Plasma VEGF-A levels were measured prior to gene transfer and at 1-6, 14, 30 and 60 days after gene transfer, using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minnesota, USA).

Statistical analysis
All data are presented as means ± SEM. Statistical analysis was performed using the two-tailed paired t test, the Wilcoxon signed-rank test, or one-way analysis of variance for repeated measures, as appropriate. With the latter, localization of differences between measurements was done with Fisher's protected least square significance test. A value of $p < 0.05$ was considered significant.

5.2 STUDY II

Study protocol
Patients with symptomatic severe coronary artery disease that could not be revascularized further were included if single-photon emission computerized tomography (SPECT) showed a significant reversible perfusion defect as estimated by two independent experienced observers. We excluded patients with an ejection fraction <0.40, unstable angina pectoris, acute myocardial infarction within the last three months, diabetes mellitus with proliferative retinopathy, diagnosed or suspected cancer, or chronic inflammatory disease.
The prespecified end points at three months follow-up were:
1) change in myocardial perfusion defects at stress and rest between inclusion and three-month follow-up SPECT studies (primary end point);
2) the safety of the percutaneous intramyocardial gene therapy;
3) changes in wall motion at NOGA mapping and contrast left ventriculography;
4) CCS angina pectoris class;
5) the frequency of anginal attacks;
6) nitroglycerin consumption;
7) patient score on the Seattle Angina Pectoris Questionnaire, and
8) exercise capacity.
Current medication was not changed until follow-up was completed. Additional clinical follow-up was performed six months after the intramyocardial injections. Signs of VEGF expression (Quantikine; R&D Diagnostics, Minneapolis, Minnesota), inflammation in terms of C-reactive protein, and VEGF-induced recruitment of CD34 stem cells (flow cytometry) were determined from successive blood determinations.
Plasmid VEGF-A165 and placebo plasmid
The plasmid VEGF-A165 was the same as in study I. The placebo plasmid was identical to the plasmid VEGF-A165 except for the VEGF-A165 gene, which had been cut out.

SPECT imaging and NOGA electromechanical mapping of the left ventricle.
Single-photon emission computed tomography studies were conducted with combined low-level exercise and adenosine infusion and injection of ^{99m}Tc-sestamibi or tetrofosmin.
With the NOGA system (i.e., NOGA mapping catheter and MyoStar injection catheter; Cordis, Johnson & Johnson, Miami Lakes, Florida), diagnostic three-dimensional maps of the left ventricle were generated for the locally measured voltage values (voltage map) and the systo-diastolic movement of the catheter tip (local linear shortening map).
Intramyocardial injections.
On the basis of the localization of the ischemic region assessed by SPECT and the local linear shortening map, the region of interest (ROI) was delineated on the NOGA map, and the injection catheter was navigated into this area. Ten 0.3-ml intramyocardial injections were given with a MyoStar mapping-injection catheter with a total dose of 0.5 mg phVEGF-A_{165} or placebo plasmid.

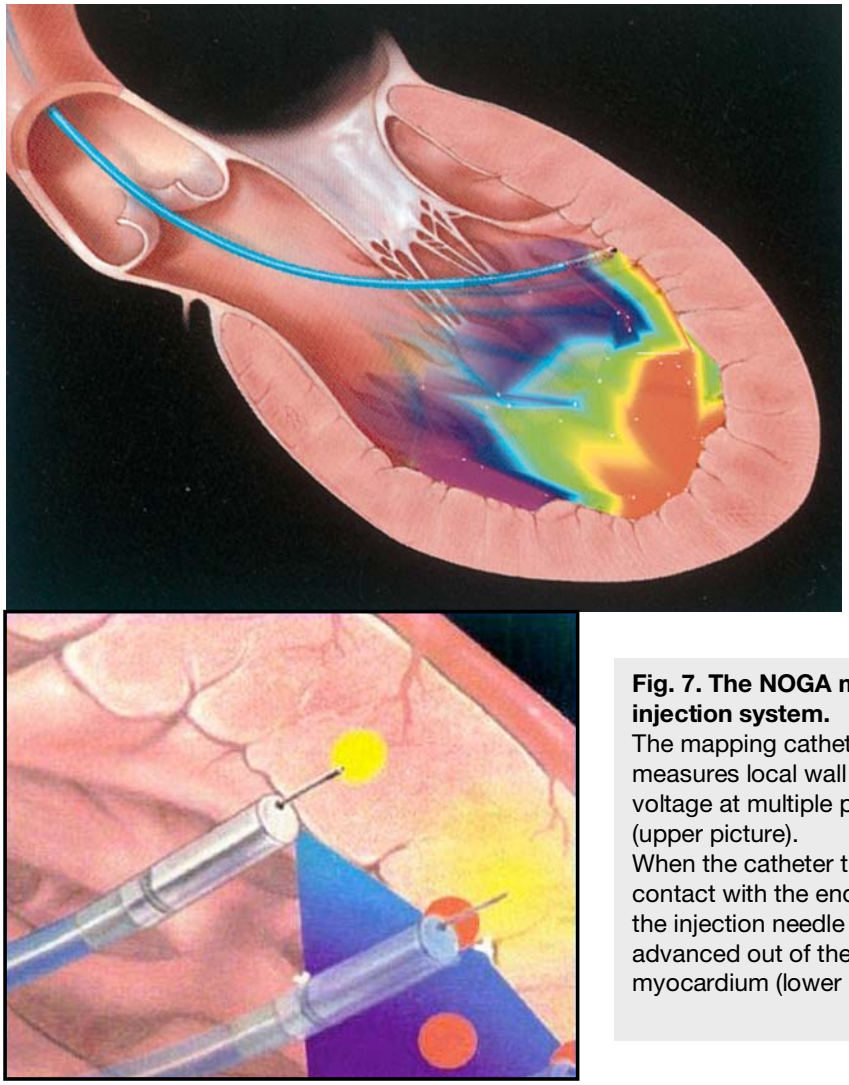

Fig. 7. The NOGA mapping-injection system.
The mapping catheter measures local wall motion and voltage at multiple points (upper picture).
When the catheter tip is in contact with the endocardium, the injection needle is advanced out of the tip into the myocardium (lower picture).

Analysis of myocardial perfusion images

For semiquantitative and visual scoring core lab SPECT analysis, the treated area (ROI) on the SPECT images was determined on the basis of the NOGA polar plot images. First, the severity of the reversible and irreversible perfusion defects at baseline was scored as defects present or defects not present as consensus readings by three experienced nuclear medicine specialists. Second, to assess changes between baseline and three-month follow-up studies, these were read together as pairs in a randomised order, which was blinded to the readers. Changes were scored as deterioration (-1), unchanged (0), and improved (+1).

Computer-based quantitative core lab SPECT analysis was made on global left ventricular perfusion. Transaxial files of the baseline and follow-up rest and stress SPECT images were analyzed with MunichHeart software (Munich, Germany). The extent of severe (normalized tracer uptake < 50%) and moderate (normalized tracer uptake between 51% and 70%) and the summarized (sum of severe and moderate) extent of rest and stress-induced perfusion defects were determined automatically and expressed as percentage of the entire myocardium.

Analysis of NOGA endocardial maps

In the quantitative core lab NOGA analysis, the ROI was delineated blinded on the basis of the injection maps at baseline and at the three-month follow-up. Researchers performed blinded quantitative assessments of the baseline and follow-up maps as mean voltage and as local linear shortening values of the ROI and remote regions.

Analysis of digitized left ventricular angiography.

At the blinded core lab analysis, the normalized motion was analyzed with the centerline method [63]. The severity of the abnormality of the regional wall motion within the left anterior descending, left circumflex coronary artery, and right coronary artery areas was computed as the mean standardized motion of contiguous chords, and it was assessed as the average standard deviation per chord (SD/chord).

Statistical analysis

The analysis was performed on the basis of intention to treat. Changes within groups between baseline and follow-up were tested using Wilcoxon's two-sided test for paired data and between groups with the two-sided Mann-Whitney U test or the exact Mann-Whitney U test. To assess differences between repeated measures between the placebo and VEGF groups, two-way analysis of variance was used. A difference was considered statistically significant at $p < 0.05$. Values are presented as mean ± SEM.

5.3 STUDY III: PROGNOSIS IN REFRACTORY ANGINA PECTORIS

This descriptive study of chronic refractory angina pectoris comprises all patients screened for the gene therapy trial EUROINJECT ONE (study II). Screening started in early 2001 and ended in July 2002. Before screening, all patients had a current clinical diagnosis of refractory angina (no further revascularisation options), based on coronary angiogram and stress tests. Screening was performed as second opinion and included single photon emission computed tomography (SPECT), echocardiography, coronary angiography and exercise test.

Patients ineligible for the trial were registered in reject logs.

Baseline data were collected from hospital records (rejected group) and the trial files (trial group). Clinical assessment of Canadian Cardiovascular Society (CCS) symptom class and exercise tests continued for 12 months in the trial. In all screened patients,

vital status was assessed by interrogation of hospital records and national population registers after a mean of 34 (range 20-47) months after screening.

Statistics

Results are presented as mean with standard deviation. Differences were tested with t-test for continuous data, Wilcoxon Signed Rank and Mann-Whitney U test for ordinal data, Fisher's exact test or Chi square test for proportions and log-rank test for survival data. A p value <0.05 was considered significant, p values >0.2 are presented as ns.

5.4 STUDIES IV–V: MYOCARDIAL GENE EXPRESSION IN STABLE ANGINA PECTORIS

Patients

Patients scheduled for coronary artery bypass surgery were selected, five for study IV and an additional three (total eight) for study V.

All had angina pectoris with stable symptoms since at least three months. In order to minimise confounding factors, subjects were not allowed to have treatment for or signs of heart failure or treatment with Angiotensin Converting Enzyme-inhibitors. For the same reason, no subject was smoking, diabetic or using cortisone or hormones. All underwent a preoperative coronary angiogram and SPECT (single photon emission computer tomography) stress-rest perfusion imaging (Software: HERMES, Nuclear Diagnostics, Stockholm). As we aimed at including patients with regional stress-induced myocardial ischemia and good angiogenic capacity, all patients had a chronic occlusion of one major coronary artery with complete filling of the distal part of the same vessel via collaterals (Rentrop score 3) [53]. Our goal was also to select patients with good adaptation to ischemia, and therefore only patients with normal systolic left ventricular function were included.

Myocardial biopsies

Two regions in the left ventricle of each subject were selected for biopsy by correlating the coronary angiogram and the SPECT images (Figure 8). The ischemic region was distal to the chronically occluded vessel on the angiogram and had a significant SPECT uptake defect at stress with normal tracer uptake at rest. Thus this region had stress-inducible ischemia and no permanent perfusion defect. The non-ischemic region served as control and was located in another part of the left ventricle without critical coronary stenosis and with normal tracer uptake both on rest and stress SPECT (no ischemia, no permanent perfusion defect).

Transmural left ventricular biopsies were obtained with a 14-gauge biopsy instrument (Tru-Core II, MD Tech, Gainesville, FL) during coronary artery bypass surgery, before cardioplegia and cross-clamping.

Biopsies were frozen in liquid nitrogen within 20 seconds and stored at –80°C.

A small part of each biopsy was formalin-fixed, paraffin-embedded, cut and stained with hematoxylin-eosin for routine evaluation. Immunohistochemical staining was performed using monoclonal mouse anti-vimentin and monoclonal mouse anti-human CD45 antibodies (DAKO, Glostrup, Denmark), with subsequent streptavidin-peroxidase incubation.

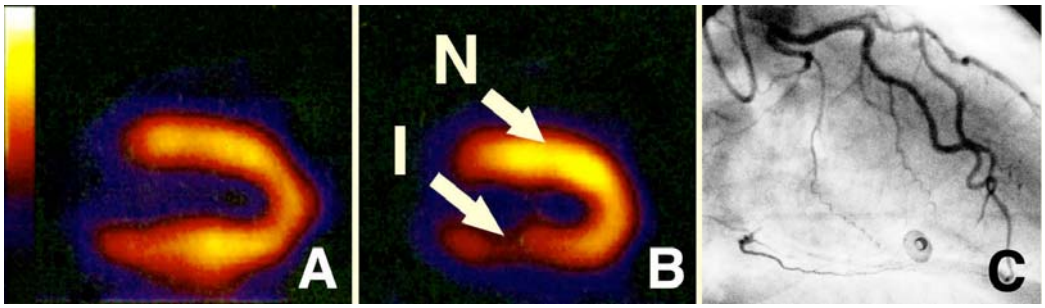

Fig. 8. Example of selected areas for biopsies. (A) Myocardial SPECT perfusion imaging, vertical long axis section, rest image: no perfusion defect. (B) Same section, stress image with perfusion defect. Arrows show the selected areas for the reversibly ischemic (I) and control non-ischemic (N) biopsies. (C) Coronary angiography in the same patient shows filling of the occluded distal right coronary artery via collaterals, explaining the stress-induced ischemia I and confirming no stenosis in the artery to the N area.

RNA procedures

Total RNA was isolated from each biopsy by the acid phenol method [93].Human Genome GeneChips oligonucleotide arrays were used (Affymetrix Inc., Santa Clara, CA), one for each sample. For the first five subjects the U95Av2 chips were used and for the last three subjects the more recent U133Plus2.0 model was used. Each of the 12624 probe sets on the U95Av2 chip was assigned a corresponding probe set on the U133Plus2.0 chip by matching for Probe set ID, UniGene ID, Gene symbol and Representative public ID. In 466 cases there was no matching probe set, leaving 12158 probe sets for analysis. The sequences of all probe sets are available at www.affymetrix.com.

Microarray data analysis

The ischemic and non-ischemic samples from the same subject were compared pairwise. Data were analyzed with Affymetrix GCOS software. The reliable detection of each probe set was determined using the "present call" algorithm, where both the absolute expression level and the background noise are taken into consideration. Change in gene expression was independently calculated in two ways: (1) Qualitative change or change call ("increase", "no change" or "decrease") and (2) Quantitative change (fold-change, i.e. times higher expression in the ischemic compared to the non-ischemic sample).

Genes were regarded as consistently differentially expressed if they had change call "increase" in at least 5 of 8 subjects and a mean fold-change of at least 1.7, or if they had change call "decrease" in at least 5 of 8 subjects and a mean fold-change below 1/1.7 (0.588). The q-value of the false discovery rate (FDR), an estimate of the false-positive probability, was calculated with SAM version 2.21 software [94]. A pairwise two-tailed t-test was used to calculate the statistical significance of differential expression for each gene, unadjusted for multiple testing.

Polymerase Chain Reaction (PCR) methods (study IV)

Quantitative real-time PCR was performed with TaqMan probes on an ABI-PRISMA 7700 Sequence Detector (Perkin–Elmer, Foster City, CA). VEGF, VEGF receptor 1 and 2, atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) were chosen as target genes. One microgram of total RNA from each biopsy was reverse transcribed by Superscript Rnase H reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexamer primers according to the manufacturer s specifications.

Amplification mixes (25 ll) contained the sample cDNA diluted, 2x TaqMan Universal PCR Mastermix, forward and reversed primers, and probe. Thermal cycling conditions included 2 min at 50°C and 10 min at 95°C before 50 PCR cycles (95°C for 15 s and 65°C for 1 min).

Change in expression was calculated as fold-change, after normalisation for beta-actin expression.

6 RESULTS

6.1 STUDY I: MYOCARDIAL DOPPLER TISSUE VELOCITY IMPROVES FOLLOWING MYOCARDIAL GENE THERAPY WITH VEGF-A165 PLASMID IN PATIENTS WITH INOPERABLE ANGINA PECTORIS

Aims
To monitor by tissue Doppler the therapeutic effects of intramyocardial phVEGF-A_{165} in patients with refractory angina pectoris.

Clinical characteristics
Six patients (two women) with stable angina pectoris without further revascularisation options were included. All used beta-blockers, aspirin and nitrates and medication was unchanged until follow-up. Details are given in Table 1.
Two patients received 1 mg and four patients 0.25 mg of intramyocardial phVEGF-A_{165} via thoracotomy.

Safety
Two patients exhibited perioperative enzyme release and one of them new Q-waves. Contributing reasons for these perioperative myocardial infarctions were the prolonged anaesthesia and operating times for the first three patients. There were no perioperative deaths.

Therapeutic effect
After two months, the maximal systolic myocardial tissue velocity improved from 6.3±0.6 to 8.0±0.6 cm/s, ($p<0.02$), in the anterior treatment area in all patients although the velocity did not reach normal values (>10cm/s). The perfusion at adenosine stress SPECT improved in the injected area in four of the six patients. The CCS angina class improved from 3.3±0.2 to 2.0±0.3 ($p<0.02$). Nitroglycerin consumption decreased from 44±17 to 15±5 tablets per week ($p<0.05$).
Plasma concentrations of VEGF-A increased 2 to 3 times ($p<0.04$) above preoperative values from 2 to 14 days after the injection.

Conclusions
The systolic myocardial tissue velocity increases in the myocardial area injected with phVEGF-A_{165}. The safety of injections via thoracotomy can be questioned.

NOTE
A twelve-month follow up has subsequently been published [95].

Patient	1	2	3	4	5	6
Age (years)	73	56	76	63	76	65
CABG (years ago)	8	10	12	15	19	12
PTCA (years ago)			4, 4, 2, 1	0.5	8	
Ejection fraction	40	55	45	45	55	45
Collateral source	LIMA–LAD	vein--> Marg	LIMA–LAD	LIMA–LAD	LIMA–LAD	LIMA–LAD
Occluded vessel	LADperiph LCx	LAD LCx	LADperiph LCx	LADperiph LCx	LADperiph Marg	Intermediate Marg
SPECT (% reversibility)	10	10	35	10	25	12
Anaesthesia time (min)	165	205	185	95	90	95
Operating time (min)	100	90	80	65	50	45
Troponin t	0,17	5,3	11	0,1	0,06	0,15
Postoperative hospital days	5	27	5	5	5	5
CCS before	3	3	3	3	4	4
CCS after	1	3	1	3	2	2
Difference before–after	2	0	2	0	2	2
NTG/week before	4	28	1	28	98	105
NTG/week after	0	14	0	16	35	22
Difference before–after	4	14	1	12	63	83
SPECT difference before–after	+	–	+	–	+	+
TVI difference before–after	+	+	+	+	+	+

CABG, coronary artery bypass surgery; PTCA, percutaneous transluminal coronary angioplasty; LIMA, left internal mammary artery; LAD, left descending coronary artery; Marg, marginal branches of coronary arteries; LCx, circumflex coronary artery; SPECT,% reversibility, single-photon emission computerized tomography,% of the left ventricle with reversibility; CCS, Canadian Cardiovascular Society functional classification; NTG, nitroglycerine tablets; TVI, tissue velocity imaging.

Table 1. Patient characteristics. Baseline, operative and two-month follow-up data.

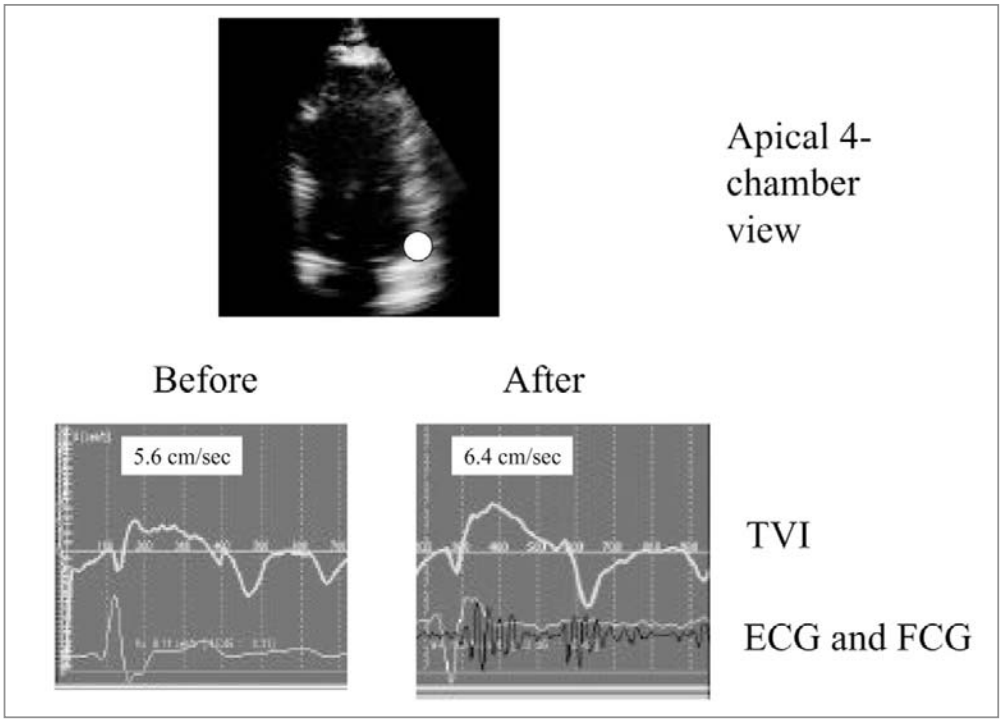

Fig. 9. Tissue velocity imaging (TVI). Dot shows basal measurement point in the phVEGF-A_{165} treated lateral wall (above). Lower row shows tissue velocity over one heart cycle during maximal dobutamine stress, before (left) and two months after (right) gene transfer. The peak systolic velocity increased from 5.6 to 6.4 cm/s. FCG, phonocardiogram.

6.2 STUDY II: INTRAMYOCARDIAL PLASMID VEGF-A165 GENE THERAPY IN PATIENTS WITH STABLE SEVERE ANGINA PECTORIS A RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED STUDY: THE EUROINJECT ONE TRIAL

Aims

To explore the efficacy of intramyocardial phVEGF-A_{165} in patients with refractory angina pectoris.

Patients

There were no significant differences in baseline characteristics (Table 2).

Safety

Three procedure-related complications occurred in the VEGF group (pericardial tamponade, temporary loss of vision, sepsis) and two in the placebo group (AV-block, myocardial infarction). In addition, during diagnostic NOGA before randomisation, one patient developed pericardial tamponade and died during emergency surgery. Seventeen major cardiac complications occurred during the six-month follow-up, not related to the gene transfer according to the independent safety committee.

Myocardial Perfusion Analysis (SPECT)

The total (whole ventricle) stress perfusion defect decreased by 10% in the VEGF group ($p=0.04$) and 5% in the placebo group ($p=0.22$) from baseline to three months The difference between groups was 1% at baseline ($p=0.73$) and 6% after three months ($p=0.18$).

Semiquantitative analysis of the treated area showed a significant ($p=0.02$) improvement in stress perfusion defects within the VEGF group, but not in the placebo group, from baseline to three-month follow-up. Again, there was no significant difference between the VEGF and placebo groups at baseline or three months.

Wall motion by NOGA mapping and left ventriculography

The local wall motion in the treated area was better after three months in the VEGF group compared to placebo (NOGA linear shortening 12.6±0.9% vs. 9.9±0.9%, $p=0.04$, Fig. 10; ventriculography -1.5±0.1 SD/cord vs. -2.0±0.2 SD/cord, $p<0.05$, Fig.11). There were no baseline differences.

Symptoms

There was a tendency ($p=0.06$) to more decrease in nitroglycerin use in the VEGF group compared to placebo. The CCS angina pectoris classification improved significantly in both groups (VEGF, from 3.0±0.04 to 2.2±0.1, $p<0.001$; placebo, from 3.1±0.05 to 2.3±0.1, $p<0.001$), with no significant difference between the groups. No significant differences between the groups were observed regarding peak exercise capacity and Seattle angina questionnaire scores.

Conclusions

Apart from catheter-related risks, percutaneous intramyocardial phVEGF-A_{165} injection is safe. Stress perfusion defects and local wall-motion improved compared to placebo. There was a tendency to more decrease in nitroglycerin use. The effect on symptoms should be tested in a larger adequately powered trial.

	Placebo	VEGF	p Value
Age, yrs	61 ± 2	61 ± 2	0.97
Gender, female/male	5/35	8/32	0.36
Diabetes, n (%)	8 (20)	7 (18)	0.77
Previous MI, n (%)	27 (68)	24 (60)	0.49
Prior CABG, n (%)	30 (75)	31 (78)	0.79
Prior PCI, n (%)	21 (52)	17 (42)	0.37
LVEF (%)	62 ± 11	61 ± 11	0.68
CCS class	3.0 ± 0.3	3.1 ± 0.3	0.55

Mean ± SEM. p value refers to differences between placebo- and VEGF transfer-treated groups.

CABG = coronary artery bypass surgery; CCS = Canadian Cardiovascular Society classification of angina pectoris; LVEF = left ventricular ejection fraction; MI = myocardial infarction; PCI = percutaneous coronary intervention; VEGF = vascular endothelial growth factor.

Table 2.
Baseline characteristics of the study group.

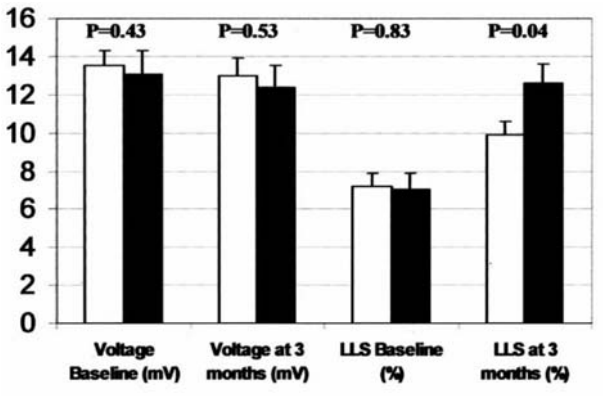

Fig. 10.
Voltage and linear local shortening in the injected area at baseline and three months. VEGF group filled bars, placebo empty bars.

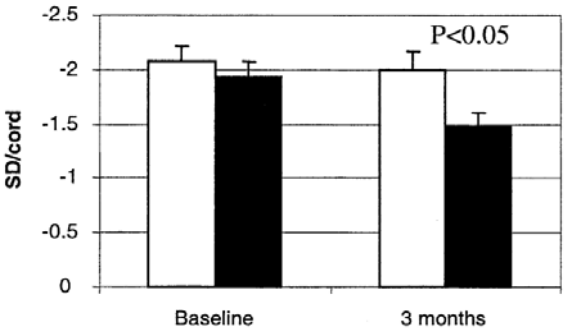

Fig. 11.
Local wall motion by left ventriculography in the injected area. VEGF group filled bars, placebo empty bars. Shorter bars indicate better function.

NOTE

A quantitative analysis of the treated area [96] showed a significantly lower amount of reversible ischemia at three months in the VEGF group compared to placebo.

6.3 STUDY III: FAVOURABLE PROGNOSIS IN REFRACTORY ANGINA PECTORIS A THREE-YEAR FOLLOW-UP OF 225 PATIENTS

Aims

To investigate the prognosis (mortality, new revascularisation options, symptomatic improvement) in patients with a diagnosis of refractory angina pectoris.

Baseline characteristics

225 screened patients, of which 80 were from the EUROINJECT ONE trial (study II) and 145 were from reject logs.

At baseline mean age was 63 ± 9 years and mean LVEF was 51 ± 12%. 10% of patients had a LVEF below 40%. The CCS symptom class is shown in Fig. 12. 22% were smokers, 23% were diabetics and 84% were previously revascularised. Coronary bypass surgery had previously been performed once in 48% and twice or more in 16%. In 59% there was a history of myocardial infarction.

Revascularisation

Although all patients had a diagnosis of refractory angina, baseline coronary angiography revealed new revascularisation options in 23 cases (10%). Six of these patients underwent bypass surgery and 17 PCI.

Among the 80 patients included in the EUROINJECT ONE trial, the protocol-specified coronary angiogram after three months revealed a new revascularization target (a new stenosis or progression of a previously non-significant lesion) in five patients, which were treated by PCI.

Mortality

Mortality was 4.1% at one year, 7.5% at two years and 10.6% at three years follow-up (fig 13). In univariate analysis, higher age ($p<0.0001$) and no use of betablocker ($p=0.023$) were associated with mortality at long time follow-up.

Using the above variables in multiple stepwise forward logistic regression, only higher age ($p=0.0007$) remained a significant independent predictor of mortality at long time follow-up.

Symptoms and exercise capacity

CCS class improved in most patients in the trial (difference to baseline $p<0.001$ for all time points) (fig 12). There was no significant difference between active and placebo groups at any time point. After three months, 31% had improved one CCS class and 23% two CCS classes or more. After twelve months the corresponding figures were 22% and 36%.

The peak exercise time increased from 474 ± 20 seconds (mean ± SEM) at baseline to 514 ± 22 after one month ($p=0.028$), 497 ± 20 after six months ($p=0.07$) and 494 ± 22 (ns) after twelve months. There was no significant difference between active and placebo groups at any time point. After twelve months, 37% had increased their exercise time by at least 60 seconds compared to baseline.

Conclusions

Patients with a clinical diagnosis of refractory angina pectoris have a rather favourable prognosis. The mortality is slightly higher than uncomplicated stable angina pectoris. Symptomatic improvement is common and new revascularisation options arise in a number of patients already within a year.

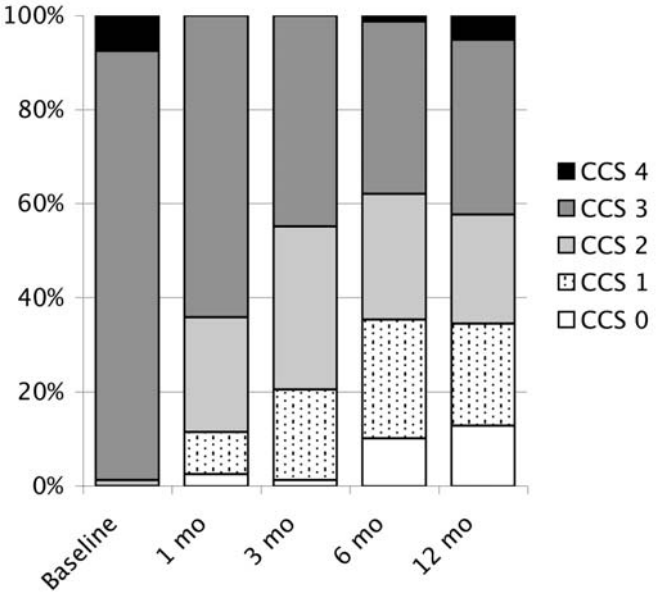

Fig. 12.
CCS class from baseline to 12 months in 80 trial patients. Marked improvement ($p<0.001$) compared to baseline for all time points. No difference between active and placebo groups.

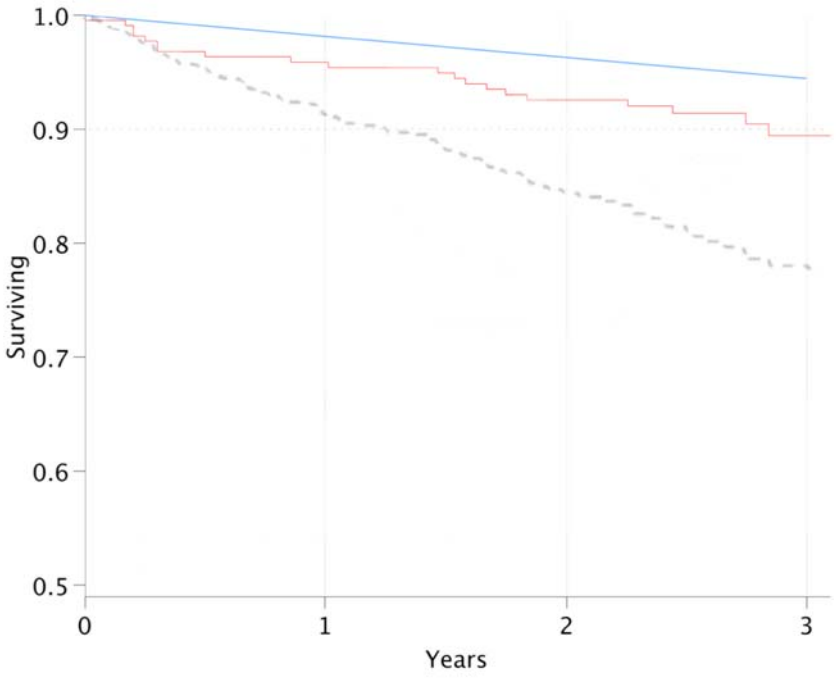

Fig. 13. Kaplan-Meier survival plot. Data from paper III in red, for comparison survival of the placebo group in the PEACE trial of stable angina pectoris with normal or slightly depressed left ventricular function [97] (blue) and in the defibrillator group in the MADIT-II trial of reduced left ventricular function after myocardial infarction [98] (dotted line).

6.4 STUDY IV: ANP AND BNP BUT NOT VEGF ARE REGIONALLY OVEREXPRESSED IN ISCHEMIC HUMAN MYOCARDIUM

Aims

To investigate the expression level of several angiogenesis-related genes, including ANP and BNP, in reversibly ischemic myocardial areas in stable angina pectoris.

Subjects

Five patients with stable angina pectoris scheduled for bypass surgery. All had a chronic coronary occlusion with good collateralisation, no heart failure, and stress but not rest perfusion defects at SPECT.

ANP and BNP but not VEGF and VEGF receptor 1 and 2 are regionally overexpressed in ischemic myocardium

ANP expression measured by PCR had a mean fold-change of 8.8 (range 0.8–31) when the ischemic sample was compared to the non-ischemic sample (fig. 14). Four of five patients had a fold-change over two. For BNP the mean fold-change was 23 (range 1.3–70), with the same four patients with a fold-change over two. VEGF-A expression had a mean fold-change of 0.9 (range 0.6–1.3). For VEGF receptor 1 and 2 the mean fold-change was 0.9 (range 0.4–1.7) and 0.9 (range 0.7–1.2), respectively.

By oligonucleotide microarray measurements, there was qualitative overexpression (change call ‘‘increase’’) in four patients for ANP, all five patients for BNP, one for VEGF-A, and two for VEGF receptor 2 (Table 3). Mean fold-change values by microarray were 4.0 for ANP, 9.9 for BNP, 1.1 for VEGF, and 1.4 for VEGF receptor 2. VEGF receptor 1 had present call ‘‘absent’’ in all patients due to low signal and therefore expression change measurement was not possible by microarray (Table 3).

Expression of 15 other angiogenesis-related genes (measured by microarray)

Due to low expression, nine genes had present call ‘‘absent’’ and expression change measurement was not possible (Table 3). Of the remaining genes, four (fibroblast growth factor 1, tumor necrosis factor a, VE-cadherin, and VEGF-B) had a mean fold-change between 1.0 and 1.2 and showed qualitative change in one or no patient. Insulin-like growth factor 1 had qualitative change in two patients and mean fold-change 1.4. Ephrin B2 had the largest change of the 15 genes: qualitative change in three patients but the mean fold-change was still only 1.4.

Histology and immunohistochemistry

Light microscopy showed essentially normal myocardium in all biopsies.

Conclusions

The overexpression of ANP and BNP in areas with stress-inducible ischemia in patients with stable angina pectoris may explain the relative inefficiency of angiogenesis. The lack of overexpression angiogenic genes (such as VEGF) supports the concept of therapeutic overexpression of these genes.

Expression of angiogenesis-related genes in ischemic compared to non-ischemic samples measured by oligonucleotide microarrays

Gene	Sequence derived from GenBank Accession No.	Affymetrix probe set number	Patients with change call "increase"	Patients with change call "decrease"	Mean fold-change
Atrial natriuretic peptide	AL021155	36663_at	4	0	4.0
Brain natriuretic peptide	AL021155	39215_at	5	0	9.9
Ephrin B2	AI765533	34335_at	3	0	1.4
Fibroblast growth factor 1 (acidic)	X59065	996_at	1	0	1.0
Insulin-like growth factor 1	X57025	1501_at	2	0	1.4
Tumor necrosis factor α	X02910	1852_at	0	0	1.1
VE-cadherin	X79981	37196_at	1	0	1.2
VEGF-A	AF024710	1953_at	1	0	1.1
VEGF-B	U48801	1926_at	0	0	1.0
VEGF receptor 1	S77812	1567_at	ND	ND	ND
VEGF receptor 2	AF035121	1954_at	2	0	1.4

Patients with neither change call "increase" nor "decrease" were "no change." ND, not detected (present call is "absent" in one or more patient samples); VEGF, vascular endothelial growth factor.

Table 3.
Gene expression by microarray. Only ANP and BNP have consistent expression changes. Nine additional genes (angiopoietin 1 and 2, fibroblast growth factor 2, monocyte chemotactic protein 1, matrix metalloproteinase 9, placenta growth factor, platelet derived growth factor b, Tie-2, and VEGF-C) had present call ''absent'' and expression change measurement was not possible.

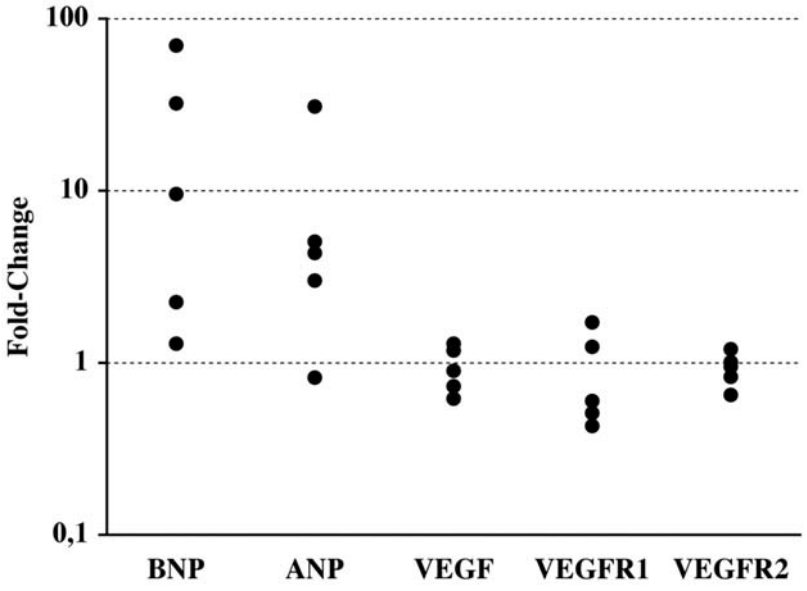

Fig. 14.
Gene expression by real-time PCR. BNP and ANP (but not VEGF, VEGF receptor 1 and VEGF receptor 2) had large expression increases (fold-change) in the ischemic area compared to the non-ischemic area. Logarithmic scale.

6.5 STUDY V: THE GENE EXPRESSION PROFILE OF STABLE ANGINA PECTORIS IN HUMAN MYOCARDIUM

Aims
To describe the gene expression pattern in reversibly ischemic myocardial areas in stable angina pectoris.

Subjects
Eight patients scheduled for bypass surgery (table 4). All had a chronic coronary occlusion with good collateralisation, no heart failure, and stress but not rest perfusion defects at SPECT.

Gene expression (by microarray)
The expression of 24 genes was consistently higher in the reversibly ischemic area than in the control non-ischemic area (table 5). They can broadly be categorized as Growth factors or related (7 genes), Muscle and structural (4 genes), Extracellular matrix (ECM, 3 genes), Coagulation related (3 genes) and Stress-responsive (2 genes) and Other function (5 genes). Nine of the overexpressed genes have a known pro-angiogenic function, four are anti-angiogenic and one is anti-apoptotic without certain angiogenic role. Three genes showed lower expression in the reversibly ischemic area. They belong to the Muscle and structural, Coagulation and Other categories, respectively. One gene is known as pro-angiogenic.

Histology and immunohistochemistry
Light microscopy showed essentially normal myocardium in all biopsies.

Conclusions
The absence of overexpression of the classical angiogenic genes and the increased expression of several anti-angiogenic genes might explain the premature plateau in collateral growth, despite the remaining ischemic stimulus. The increased expression of several anti-apoptotic and muscle-related genes might explain the preserved left ventricular function even after a total coronary occlusion.

Subject number	1	2	3	4	5	6	7	8
Age	63	60	61	53	71	52	52	72
Ischemic area	inferior	inferior	inferior	inferior	anterior	inferior	inferior	inferior
Occluded Vessel	RCA	RCA	RCA	LCX	LAD	RCA	RCA	RCA
Normal area	anterior	anterior	anterior	anterior	lateral	anterior	anterior	anterior
Symptom class	2	2	2	3	3	2	2	3
Angina duration (years)	0.5	0.5	1	0.3	2	1	0.5	12
LVEF %	68	69	55	48	69	58	50	69

Table 4. Patient characteristics. RCA, right coronary artery; LCX, left circumflex coronary artery; LAD, left anterior descending artery. LVEF, Left Ventricular Ejection Fraction (assessed by gated single photon emission computed tomography).

Gene symbol	Gene name	No of increase calls	Mean fold change	FDR q	Significance p	Functional group
Pro-angiogenic						
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	8	3.0	0.24	0.06	Other
HSPA2	heat shock 70kDa protein 2	8	2.2	0.53	0.036	Stress
CTGF	connective tissue growth factor	7	2.5	0.24	0.0028	Growth
CNN1	calponin 1 basic smooth muscle	7	2.5	0.62	0.037	Muscle
LTBP2	latent transforming growth factor beta binding protein 2	6	2.4	0.51	0.0052	Growth
F2R	coagulation factor II (thrombin) receptor	6	2.3	0.64	0.0034	Coagulation
CSPG2	chondroitin sulfate proteoglycan 2 (versican)	6	1.9	0.64	0.023	ECM
BGN	biglycan	6	1.8	0.64	0.047	ECM
FAP	fibroblast activation protein alpha	5	2.2	0.24	0.0015	Growth
Anti-angiogenic						
NPPB	natriuretic peptide precursor B	8	9.2	0.57	0.05	Growth
IGFBP3	insulin-like growth factor binding protein 3	7	2.0	0.43	0.015	Growth
NPPA	natriuretic peptide precursor A	5	3.4	0.54	0.039	Growth
SERPINE2	serine proteinase inhibitor clade E (nexin) member 2	5	1.7	0.82	0.049	Coagulation
Anti-apoptotic, Angiogenic function uncertain						
IER3	immediate early response 3	7	2.0	0.58	0.023	Stress
Uncertain function in angiogenesis and apoptosis						
THBS4	thrombospondin 4	8	2.2	0.24	0.00046	Other
MLLT11	myeloid/lymphoid or mixed-lineage leukemia; translocated to 11	7	2.9	0.64	0.053	Other
MXRA5	matrix-remodelling associated 5	7	1.9	0.24	0.0018	ECM
DOK5	docking protein 5	7	1.8	0.92	0.036	Other
TNNT1	troponin T1 skeletal slow	6	3.1	0.51	0.026	Muscle
PROS1	protein S (alpha)	6	2.0	0.79	0.036	Coagulation
TPM3	tropomyosin 3	6	1.8	0.24	0.011	Muscle
DIO2	deiodinase iodothyronine type II	5	3.7	0.62	0.053	Other
AP2B1	adaptor-related protein complex 2 beta 1 subunit	5	2.8		0.017	Growth
MYH10	myosin heavy polypeptide 10 non-muscle	5	1.8	0.58	0.097	Muscle

Table 5. Overexpressed genes. Genes with change call "increase" in at least 5 of 8 subjects and with mean fold change of at least 1.7. Presented by known angiogenic function, then ordered by number of increase calls. FDR q is the q value of the False Discovery Rate. Significance p is the p value calculated with a paired two-tailed t-test. Functional groups, see text.

7 GENERAL DISCUSSION

7.1 THERAPEUTIC ANGIOGENESIS WITH PHVEGF-A165

The key question in this thesis is how to enhance the naturally occurring but limited angiogenesis and collateral growth in coronary heart disease. The safety and efficacy of one therapeutic agent, intramyocardial plasmid VEGF-A_{165}, is described in the first two papers.

7.1.1 Safety

There were no signs of systemic (such as tumour growth, inflammatory disease or retinopathy) or myocardial side effects of phVEGF-A_{165} in any of the two studies, with 46 patients receiving active treatment. This confirms previous studies where about 40 patients have received intramyocardial phVEGF [26, 90, 92, 99].
The delivery via thoracotomy was associated with myocardial infarction in two patients, one of which impaired the left ventricular function. This was not reported in a similar trial from Boston [92]. Both in Boston and in our trial program, the thoracotomy method was abandoned in favour of the percutaneous NOGA mapping-injection catheter system. Among the 80 patients in study II, one developed a myocardial infarction after the injection procedure and two developed a pericardial tamponade (one fatal, albeit after diagnostic mapping before randomisation). Three other less severe catheter-related complications occurred. There is probably a learning curve, with increased safety with increased experience. *Altogether, the percutaneous system in the current version seems safer but still induces a significant amount of serious complications.*

7.1.2 Effect on perfusion

Perfusion on stress-rest SPECT in study II was assessed at baseline and three months follow-up. Data were analysed in two ways in study II. The semiquantitative visual scoring of the treated area showed that more patients in the VEGF group improved than in the placebo group. The presence of perfusion defects at stress SPECT was not different between the groups; however this only included scores for defect present or absent and thus does not include any assessment of defect size or reversibility.
Secondly, a computer based analysis of the perfusion defects in the whole left ventricle was performed. When severe and moderate defects were added, there was a 6% lower defect size in the VEGF group compared to the placebo group after three months ($p=0.18$). The difference from baseline to three months was statistically significant in the VEGF group ($p=0.04$), but not in the placebo group.
The reversible perfusion defect, that is the difference between stress and rest perfusion defects, might be a more logical endpoint as this reflects the amount of ischemia. A quantitative analysis of the reversibility in the treated area was not possible in the main manuscript (study II) because of difficulties in transforming the coordinates of the treated area from NOGA to SPECT.
Therefore, a more detailed analysis of the SPECT and NOGA mapping data was published separately [96]. The treated area amounted to about 20% of the entire left ventricle. Within the treated area, the reversible ischemia at three months was reduced

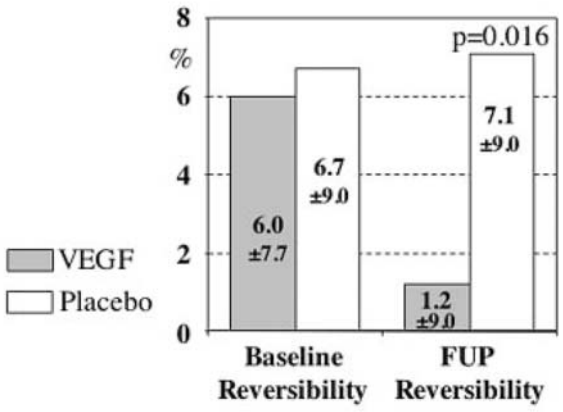

Fig. 15. Study II: reversible perfusion defects on SPECT decreased significantly in the VEGF group (p=0.016 for difference between groups at three months follow up).

to 1% in the VEGF group and remained at 7% in the placebo group (p=0.016 for difference between groups). Thus this confirmed the 6% difference in global perfusion defects in manuscript II. There was no change in the untreated parts of the left ventricle.
Is this 6% decrease in reversible perfusion a clinically meaningful result?
In study II, this represents that 80% of the reversible ischemia was abolished, so it would be unrealistic to demand more. A decrease in perfusion defects at rest, which usually are interpreted as infarcted area, was not seen in this trial, as expected.
The AGENT-2 trial with intracoronary adenovirus encoding FGF-4 [89] was considered positive. However, the reversible perfusion defect size decreased from 20% at baseline to 16% at follow-up, a 21% relative improvement, less than in study II.
The KAT trial [91] investigated liposomal plasmid VEGF, adenoviral VEGF or placebo, delivered with a coronary perfusion catheter during PCI. The reversible perfusion defect size on SPECT decreased from 8% to 2% with adenoviral VEGF and from 11% to 6% with placebo. The effect in study II was thus comparable to the KAT trial, but the latter included PCI in addition to gene transfer. This is reflected in the similar improvement in the placebo group in KAT but not in study II.
In an unblinded but controlled trial of the same product and delivery route as study II [99], PET perfusion improved at stress by about 30% in the ischemic area.
Several other treatments which are used in patients with chronic angina pectoris have shown no significant effect on SPECT reversible perfusion defects: laser revacularisation [19, 21], enhanced external counterpulsation (EECP) [18], spinal cord stimulation (SCS) [100]. The metabolic modulator trimetazidine has been reported to improve both stress and rest perfusion defects but not reversible defects [101]. The addition of a calcium antagonist to a betablocker (or vice versa) is common in clinical practice but has not been shown to improve SPECT perfusion [102].
Thus, intramyocardial phVEGF-A_{165} improved reversible perfusion defects.

7.1.3 Effect on local wall motion

In study I, wall motion in the treated area increased by about 25% at stress tissue doppler echocardiography. These findings were confirmed in study II, where local wall motion improved (at rest) both on left ventriculography and on NOGA mapping. These effects where somewhat unexpected, and in fact few angiogenesis trials have measured this parameter. This local improvement did not translate into any measurable improvement in global left ventricular systolic function.

Nevertheless, the effect on wall motion is interesting. It might indicate that areas with decreased systolic function but remaining viability (hibernation) are ideal targets for angiogenic gene therapy. The decision to exclude patients with depressed left ventricular function from study II may have limited the number of ideal patients in study II and thus limited its power. In the future, perfusion imaging techniques such as PET could elucidate the effect on hibernation further by absolute perfusion measurement and better spatial resolution.
Thus, intramyocardial phVEGF-A_{165} improved local wall motion.

7.1.4 Symptomatic effects

The effect on symptoms was not the primary endpoint of study I or II, and neither study had been power-calculated for these endpoints. Even though study II was double-blind, the exercise ECGs were not core-lab analysed, and therefore time to ST-depression was not calculated. Furthermore, there was some missing nitroglycerin and exercise test data, which decreased the statistical power.
The effect on CCS angina class was substantial in study I and study II (active and placebo groups). The mean improvement was one CCS class, with no significant difference between active and placebo at any time point up to 12 months (manuscript III).
While the decrease in short-acting nitroglycerin consumption was dramatic in study I (44 to 15 tablets per week) the effect was smaller in study II (19 to 11 tablets per week in the active group). Unfortunately, there was a slight imbalance in the groups at baseline (12 vs. 19 tablets per week in the placebo group and active groups, p=0.13). After three months there was no significant difference between the groups (p=0.15). It might be better to calculate the difference in nitroglycerin use between baseline and three months follow-up, as this discards the patients with missing data at baseline or follow-up. Thus, the mean decrease in nitroglycerin use from baseline to 3 months was 10 in the active group (p=0.009 for difference to zero) and 0.1 (p=0.87) in the placebo group. The difference between groups was borderline significant (p=0.06).
The exercise test protocol used 25 watt steps every 2 minutes, which makes the analysis of exercise capacity in watts rather crude. After three months there was no difference between active and placebo groups (108 vs. 112 watts). The exercise time in seconds was calculated in manuscript III, and the mean value increased by 20-40 seconds up to

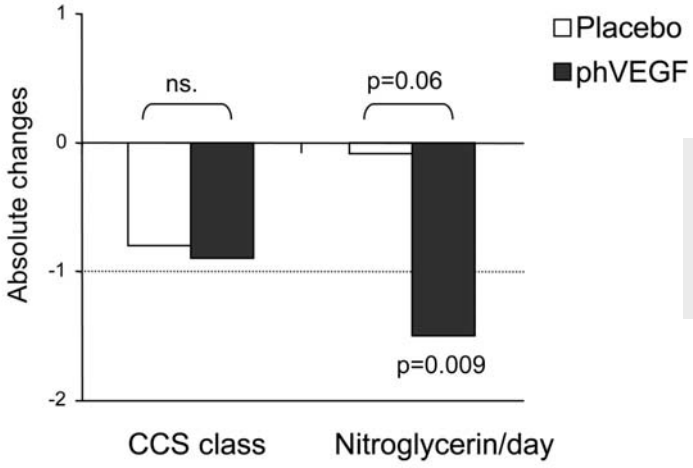

Fig. 16. Study II: difference in symptoms from baseline to three months. Negative values indicate improvement.

one years follow-up (placebo and active groups combined). The increase was only statistically significant at one month (p=0.028). A significant number of patients (37%) increased their exercise time by more than 60 seconds at one year follow-up. There was no difference between placebo and active groups at any time point.
Thus, there was a tendency to decrease in nitroglycerin use in the VEGF group compared to placebo, but no other differences between the groups. The symptomatic effect should be tested in a larger adequately powered trial.

7.1.5 Placebo effects

There was a profound placebo effect in the CCS angina class, a small placebo effect on exercise test but somewhat surprisingly none in nitroglycerin use, SPECT perfusion and local wall motion. One explanation might be that both patient and investigator tend to be biased towards an improvement after treatment. In a blinded randomised trial this effect is the same in both groups but leads to an improvement over time (or placebo effect). For CCS angina class, which is usually scored by the investigator and not the patient himself, one trial found that 28% of the improvement was due to investigator bias [20]. Again, in a placebo-controlled trial this effect will occur in both groups. With exercise test there could also be both patient bias and investigator bias (i.e. encouraging the patient more), but with nitroglycerin consumption there should only be patient bias, as this was self-reported in writing. The presence of investigator bias might thus explain that the placebo response was large on CCS class, smaller on exercise test and none on nitroglycerin consumption.
In contrast, SPECT and local wall motion was assessed at core labs blinded to the before and after treatment status of the data, thus eliminating bias. Other double-blind

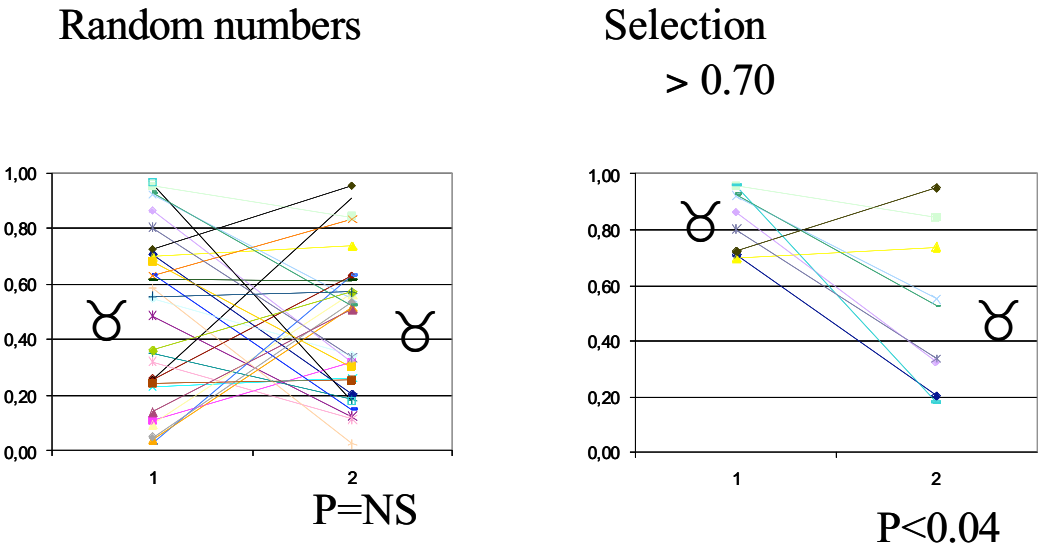

Fig. 17. Example of regression to the mean. Random numbers from 0 to 1 are sampled twice and connected in pairs, the mean values are similar at both occasions and the difference is not statistically significant (left panel). From the same numbers, values above 0.70 are selected at the first occasion, as an example of an inclusion threshold in a trial. At the second occasion, the corresponding values have a mean close to the mean of the whole group, and statistically different from the selected group at the first occasion (p<0.04).
In clinical trials this effect will be smaller but measurable as the variables have a smaller random variation than in this illustration.

trials have found small or no placebo effects on SPECT while sustained and substantial symptomatic improvement with placebo has been seen on exercise tests, CCS class and nitroglycerin use in several trials [21, 103].
Another explanation to improvements in trials is the regression to the mean effect. If a subgroup is selected by taking the higher part of the distribution of a variable, the mean value in the subgroup will decrease from baseline to follow-up. If the lower values are selected, the mean will increase instead. In fact, it will regress towards the mean of the whole group before the selection process [104, 105]. This is true as long as there is variation in the variable, either randomly, due to measurement errors or cyclically over time.
This effect will be seen in many clinical trials, as the study participants usually are chosen after an inclusion test, and therefore are a subgroup. If the value (i.e. blood pressure) exceeds a threshold, the patient enters the trial. The same value is then measured again at follow-up. A regression towards the mean-effect should be expected. For study II this means that we would expect CCS class and reversible ischemia to decrease at follow-up solely because of this statistical effect, as both these variables were used as inclusion thresholds.
As we used placebo plasmid, and not saline, as placebo one might suspect that the empty plasmid exerted angiogenic effects on its own. Even if some data points to unspecific effects of high doses of plasmid [106], our own animal data have shown no angiogenic effect of placebo plasmid when it was compared with phVEGF-A_{165} [107]. In fact, there was no difference to saline.
Thus we argue that biologic effects of the placebo plasmid hardly can explain the improvements in the placebo group in study II. A combination of regression to the mean effects, investigator bias and patient expectations are more likely.

7.1.6 Lack of association between perfusion and clinical improvement

There were no clearly significant effects (beyond placebo) on clinical endpoints in study II, although most of the reversible ischemia was abolished according to SPECT. There seems to be a lack of association between improved perfusion and improved overall function of the patient. In a PET study the correlation between symptomatic improvement and perfusion improvement was weak [99]. Another study found no association between the patients symptoms and inducible ischemia on SPECT [108]. A similar example is a study where patients one year after one-vessel PCI did not improve their maximum exercise capacity despite being relieved of clinical angina and perfusion defects [22, 23].
It seems plausible that many patients with coronary artery disease have other limiting factors besides myocardial ischemia, especially in refractory angina. Skeletal muscle deconditioning because of lack of physical exercise and chronic pain disorders are examples [109].
The impact of improved myocardial perfusion on the overall physical ability of patients seems surprisingly small.

7.1.7 How to achieve a better clinical effect

So far, no angiogenic agent has been shown to be superior to placebo on clinical endpoints in ischemic heart disease. The therapeutic effect depends on the combination of the delivered gene, the vector, the delivery route and the treated patient group. This

gives many combinations, many still unexplored in clinical trials. In addition, there is the question of a meaningful and measurable endpoint.

7.1.7.1 Which gene

Among single angiogenic factors VEGF is probably the most extensively studied for ischemic diseases. It has been shown to improve myocardial perfusion in animal models [25, 74, 75, 79, 110]. Small uncontrolled studies [26, 92] (including study I) showed a dramatic decrease in nitroglycerin need and decreased ischemia. Paper two is the first and only double-blind placebo-controlled randomised trial with intramyocardial phVEGF-A_{165}.

Would another growth factor have been be better?

Available data for FGF do not show better effects than for VEGF. The large clinical trials AGENT-3 and 4 (with intracoronary adenoviral FGF-4) were terminated by the sponsoring pharmaceutical company Schering because no therapeutic effect was seen. In the previous smaller trial AGENT-2 a small non-significant effect on perfusion was seen [89]. Possible reasons include an inefficient delivery method (intracoronary) and a FGF variety with rather weak documentation in animal models.

It might be advantageous to use an upstream factor, such as the regulatory gene HIF-1α, which is activated by ischemia and induces several angiogenic genes besides VEGF. Positive effects have been shown with adenoviral HIF-1α in porcine models of myocardial ischemia [87] and with plasmid HIF-1α in the ischemic hindlimb model [111]. It remains to be seen if these effects can be reproduced in clinical trials.

Subcutaneous injections of the stem cell releasing factors G-CSF and GM-CSF might also have therapeutic potential, indicated by small clinical trials [112-114]. There have however been safety concerns with restenosis and possible destabilisation of plaques [115, 116]. Recently, a randomised trial after myocardial infarction showed no benefit of G-CSF on systolic function [117].

Another concept is combination therapy with several growth factors. Animal experiments indicate that combinations with VEGF and Angiopoietin-1 [77] or FGF and PDGF [118] might enhance angiogenesis. The potential of side-effects might increase with more powerful stimulation. Excessive stimulation of angiogenesis might include retinopathy, tumour growth and even local hemangioma formation [119]. This has however not been seen with the mentioned combinations.

Lastly, it seems plausible that the therapeutic effect of any angiogenic gene therapy would be potentiated by increased physical exercise to boost the naturally occurring angiogenesis [24, 120]. Relating to that, a case report on a Swiss cardiology professor showed increased collateral flow after intense endurance training in the absence of coronary stenosis [121].

Thus, VEGF seems to be a good choice.

7.1.7.2 Which vector

The transfection efficiency of a plasmid is low but with intramyocardial delivery enough to produce VEGF protein and induce angiogenesis and arteriogenesis in animal models [25, 75-79]. Adenoviral vectors have a higher transfection efficiency, but they elicit a local inflammation which may cause safety problem at intramyocardial delivery. If a higher protein expression with viral vectors also translates into more angiogenesis is uncertain. In a study of HIF-1α adenoviral vector was more efficient than plasmid but also induced more inflammation [87]. In a study from our own institution adenoviral VEGF induced a higher protein expression than its plasmid counterpart

[122], but the angiogenic potency was the same. The angiogenic effect of VEGF might have a ceiling, and protein levels over this ceiling might not cause better clinical effects.
Thus, plasmid seems to be a good choice for intramyocardial delivery.

7.1.7.3 Which dose

In study I two patients were injected with 1.0 mg and four patients with 0.25 mg of phVEGF-A_{165}. In study II a dose of 0.5 mg was used. Would a higher dose have been more efficacious?
The doses were selected after the experiences in Boston where 0.125-0.25 mg [92] and 0.2-2.0 mg [90] of plasmid has been used in trials. In porcine models, 0.5 mg [25] and 1 mg [75] have been used. In a rat model a dose response in VEGF expression was found from 3-30 µg but not with higher doses [78]. The rats weighed about 400 grams, about 200 times less than a human being. Thus the optimal dose in the rat study, 30 µg, corresponds to about 6 mg in a human. Of course metabolic species differences make this calculation only approximate. In all the above studies no adverse effects of the plasmid have been noticed.
There is however a possibility of dose-limiting local side effects also with plasmids. An inflammatory response has been noticed after injection of 25 µg of plasmid into the tibialis muscle of mice [106], thus a dose much higher than what we used in the trials. This inflammation might be caused be unmetylated CpG-motifs in the plasmid backbone itself. Angioma-like vessel formation was reported after 500 µg of VEGF plasmid in an ischemic rat model [123], possibly caused by excessive VEGF expression. Again, this was a very high dose, over 100-fold higher than in our trials. Animal data from our lab with therapeutic doses of placebo plasmid showed no difference in angiogenic response to saline, thus ruling out unspecific effects of the empty plasmid .[107]
In conclusion, it seems safe to increase the dose up to at least 2 mg in humans. If there is a dose response above that level in the angiogenic effect (and not only in VEGF protein expression) is still not clear.

7.1.7.4 Which delivery route

Intracoronary injection is easy to perform with standard equipment in the cath lab but gives both low myocardial expression and high systemic spill-over. It is unlikely that intracoronary injections of a plasmid give higher transfection of the myocardium than the other organs. Viral vectors have in animal trials shown some effects with intracoronary injection, but the induced protein was at least tenfold lower compared to intramyocardial injection [124]. In one study, only 0.88% of the growth factor was found in the myocardium after intracoronary injection [125] while this value was 18% after intramyocardial injection [126]. Direct intramyocardial injection thus appears preferable. It can be performed either with a surgical approach, or more recently with a percutaneous catheter system.
The surgical approach resulted in perioperative myocardial infarction in two of six patients in study I. Even though other studies with the same technique had less adverse events [92], this indicates an unacceptable safety profile with surgical injections, especially if the rather low mortality of patients with refractory angina is considered (study III). This was not known at that time, however.
With the advent of the NOGA injection-mapping catheter system, the surgical procedure became obsolete [127, 128]. The percutaneous technique is as efficient as the

surgical technique [126]. Studies have shown a optimum delivery volume of 0.1 ml per spot with a needle length of 6 mm in pigs [126, 129]. In study II, we used quality control criteria for injections, which ensure successful intramyocardial injections in 95% of cases [128].
In conclusion, the used percutaneous injection technique in study II seems to be the best technique available.

7.1.7.5 Which patient population

Papers one and two targeted patients with refractory angina pectoris, most of them with recurrent symptoms years after bypass surgery. They had limiting symptoms despite optimised medication. It would seem challenging to succeed in these patients, where all other therapies have failed. Factors such as diabetes [42], hypercholesteremia [43-45] and higher age [47, 48] have indeed been shown to impair the angiogenic response.
It might be more promising to apply angiogenic treatment earlier in the time course of coronary artery disease. In stable patients, prognostic benefits of revascularisation are expected only in patients with large ischemic areas or severe proximal multivessel stenosis [130]. In other stable patients, revascularisation offers just symptomatic relief, and angiogenic gene therapy might be investigated. The risks associated with a percutaneous gene therapy procedure are probably smaller than with bypass surgery, which is the other option when PCI is not possible.
A suitable patient category might be coronary chronic total occlusions, where PCI is not successful in 40% of patients [131].
As studies I and II showed improved local wall motion in the treated ischemic area, it seems logical to include patients with a large zone of compromised systolic function, but with remaining viability *(hibernation)*.

7.1.7.6 Which endpoint

Most trials on angiogenic gene therapy have been performed as academic studies with limited funding. Larger trials with hundreds of patients are hardly possible as academic studies. Endpoints in studies like study II with 80 patients must be chosen with care. The endpoint should be clinically meaningful, measurable with small standard deviation and sensitive to change.
Measurements of myocardial perfusion or mechanical function (discussed in detail in section 3.2.3) are sensitive to change and provide evidence of biological effect of the gene transfer. They are therefore well suited as surrogate endpoints in smaller trials, such as study II, but in order to finally prove the clinical utility of a treatment for chronic angina pectoris, a clinical endpoint must be used in a larger trial.
Maximal exercise capacity at a treadmill test or bicycle ergometry has been used as an endpoint in many studies. Care should be taken to minimise errors like different time of day and variable encouragement by the test supervisor. Taking the mean of two exercise tests on consecutive days might reduce the standard deviation.
The time to ST-depression or time to chest pain might be more sensitive to changes in angina status [22], whereas the maximal exercise capacity is determined also by other factors (skeletal muscle function, pulmonary disease). In refractory angina pectoris, those limitations are common and the correlation between exercise capacity and myocardial perfusion changes is weak. In our experience only a minority of refractory angina patients have significant ST-depression on exercise tests but most develop chest pain. *Therefore, time to chest pain might be preferable as endpoint in refractory angina patients.*

The ultimate goal would of course be to lower mortality, but that would require a large trial over several years, as the mortality in refractory angina is rather low annually. A composite of mortality, revascularisation and myocardial infarction would require slightly less patient-years.
More relevant in refractory angina pectoris is to lessen symptoms. This could be measured as CCS angina class, where a self-administred form probably is superior to the more common grading by the physician [132].
Quality of life measurements also seem meaningful. Disease-specific questionnaires include the Seattle Angina Questionnaire (SAQ) [133], the Duke Activity Status Index (DASI) [134] and Quality of Life after Myocardial Infarction-2 (QLMI-2)[135]. Common generic scales include the Short Form-36 (SF-36) [136] and Nottingham Health Profile (NHP)[137]. These scales might have a low sensitivity to change in symptoms. One review recommended the use of QLMI-2 or SAQ plus SF-36 in patients with ischemic heart disease [138].
Thus, endpoints in study II seem relevant. Possible improvements include centrally monitored and core-lab evaluated exercise tests including time to chest pain, addition of SF-36 as a generic quality-of-life measurement, self-reported CCS angina class and improved imaging techniques (PET, stress echo with strain rate). In this academic study, the limited funding somewhat limited the possibilities.

7.2 PROGNOSIS IN REFRACTORY ANGINA

When this thesis project started, the prevailing view was that patients with refractory "end-stage" angina pectoris had a poor future, justifying a significant procedural risk in order to improve their symptoms. The EUROINJECT trial (study II) gave us the opportunity to scrutinize this view in a substudy on the whole screened population (manuscript III).

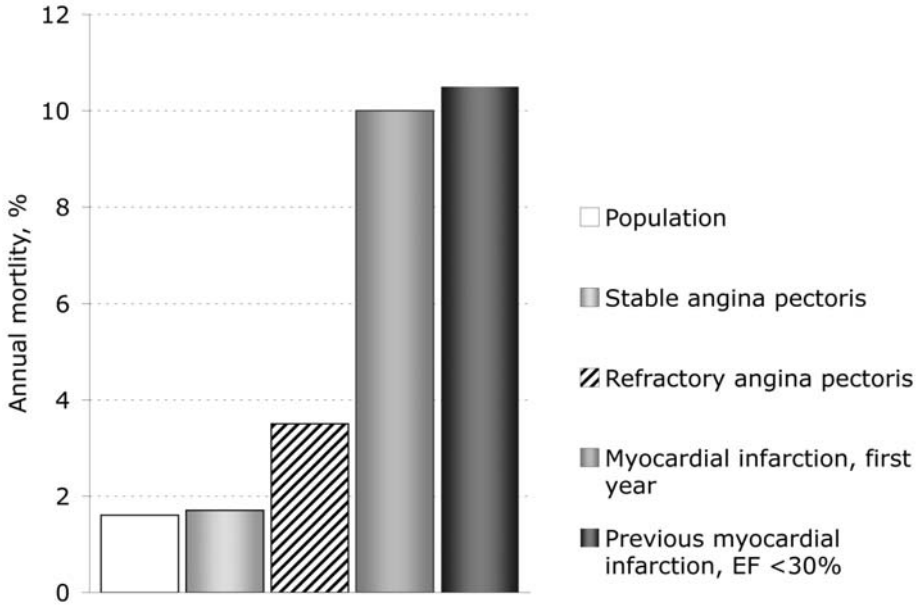

Fig. 18. Annual mortality in coronary heart disease in perspective.
Mortality in refractory angina pectoris is somewhat higher than in the background population and in stable angina pectoris in general, but considerably lower than after a myocardial infarction.

7.2.1 Mortality

The mortality in 225 patients with a clinical diagnosis of refractory angina was 10.6% after three years (manuscript III). Other smaller trials have shown higher mortality, 3-17% per year. This cannot be explained with differences in mean age, prevalence of diabetes or ejection fraction as these were similar in our material and other studies. Although SPECT was not done in all patients, we suspect that many patients had a rather small reversible perfusion defect, which may explain the rather low mortality.
Is the observed 3-4% annual mortality high?
First, the background mortality in the same age range was estimated. We picked Danish statistics as most patients were found there. In men aged the 63, the annual mortality was 1.6%.
Mortality in stable angina pectoris in recent trials has been very low (1.5-1.7% annually) [97, 139, 140]. In a recent 9 year follow-up of 809 stable angina patients, the annual mortality was 2% [141]. Only men in the first three years after diagnosis had a higher mortality than a matched reference population.
Myocardial infarction in patients aged 60-69 had a one-year mortality of 10% in the Swedish national registry [142]. In survivors of myocardial infarction (which 59% of study III patients were) with an ejection fraction of 30% or less, the annual mortality was 10,5% [98].
Thus, the mortality in refractory angina pectoris is higher than in the background population and stable angina pectoris in general, but substantially lower than in heart failure and in the first year after myocardial infarction (fig. 18).

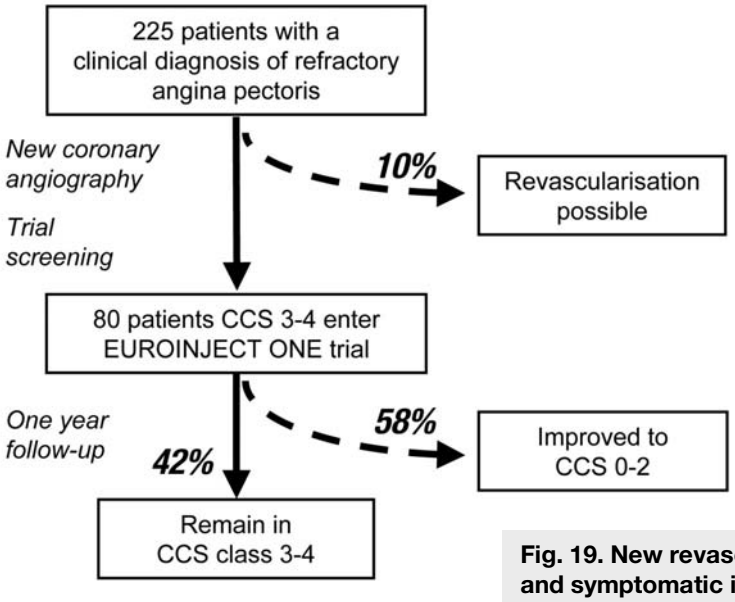

Fig. 19. New revascularisation options and symptomatic improvement are common (study III).

7.2.2 Refractory angina pectoris – not so refractory?

As mentioned in section 7.1.4, the CCS class improved substantially during one year after inclusion in the trial, both in the placebo and active groups. 36% of patients had

improved by two or more CCS classes after one year. In other words, only 42% remained in CCS class 3-4 after one year. Similarly, 37% increased their maximal exercise time by at least 60 seconds after one year. Again, there was no significant difference between active and placebo groups. Clinical improvement in placebo-treated patients with refractory angina continues for at least 2 years [103].
In the EUROINJECT trial, the protocol-specified new angiogram after 3 months found a surprising number of new significant lesions. 6% of patients were treated by PCI because of these new findings. In addition, at the baseline screening angiography, 10% of patients were possible to revascularise, although they were thought to have no such possibilities (hence the diagnosis of refractory angina).
Clearly there seems to be a potential for spontaneous symptomatic improvement, as seen in the placebo group. Furthermore the occurrence of new treatable stenosis is rather common.
Thus, a diagnosis of refractory angina pectoris should be regarded as a temporary state, not a life-long diagnosis. The patient should not be denied new coronary angiography, as a more proximal treatable stenosis may have developed.

7.2.3 No ischemia on SPECT despite refractory angina – what does it mean?

In 51 of 225 screened patients (23%) there was no clear reversible perfusion defect on SPECT, despite a diagnosis of refractory angina and multivessel coronary artery disease (manuscript III).
One explanation is that the angiogram just rates the stenosis severity without seeing the contribution of the collaterals to lessen ischemia in the myocardium. In other words, the SPECT shows physiology and the angiogram anatomy. However, contemporary coronary angiography does not stop with visual interpretation of stenosis severity. In the case of borderline stenosis, intracoronary pressure measurement (Fractional Flow Reserve, FFR) is often done and adds precise physiological information [143, 144].
The opposite view on the difference between SPECT and angiogram is that SPECT is a rather crude method with a limited predictive value for ischemia. The sensitivity and specificity is about 85% for detecting coronary stenosis [59], and in 18% even three-vessel disease is reported as no reversible perfusion defect [57, 58]. This may be due to "balanced ischemia" [145] and also to the limited spatial resolution. In fact, FFR measurement has been shown to be superior to SPECT in risk stratification of multivessel coronary artery disease [146].
It seems fair to conclude that *a negative SPECT does not preclude a diagnosis of refractory angina,* at least when there is other evidence of myocardial ischemia. This could be intracoronary pressure measurement (FFR), stress echo, MRI or PET. A negative SPECT however seems to indicate a good prognosis [59].

7.3 MYOCARDIAL GENE EXPRESSION IN STABLE ANGINA PECTORIS

Papers IV and V describe the myocardial gene expression pattern in stable angina pectoris. This may give clues to which genes could be overexpressed therapeutically to enhance angiogenesis and why collateral growth stops before if fully compensates myocardial ischemia.

7.3.1 Classical angiogenic factors are not overexpressed

In manuscript IV and V, the expression of 18 genes with well-known angiogenic function was not increased in reversibly ischemic myocardium as compared to a normal control area. The findings from microarray experiments were validated with more precise real-time PCR for VEGF, VEGF receptor 1 and 2.
Many of these genes have a steep expression increase after acute myocardial ischemia and infarction. We argue that there is a major difference between the profound ischemia, necrosis and apoptosis accompanying myocardial infarction, and the limited repetitive ischemia in stable angina pectoris. The remaining intermittent ischemic stimulus in our stable angina patients was proven by SPECT imaging and symptoms. *It seems that after weeks or months of intermittent myocardial ischemia, the initial angiogenic gene expression response has faded out.* This observation has also been made in animal models [35].
Interestingly, the plateau of collateral growth is also reached after a few weeks to months [28, 68]. So, does the faded gene expression response cause the cessation of collateral growth? Or has the growth of the collateral arteries diminished the ischemia under a threshold, required for increased expression of VEGF and other angiogenic factors? Or has the expression of angiogenic genes been limited by increased expression of inhibiting factors after a few weeks? The answers to these questions are not completely clear.

7.3.2 ANP, BNP and other angiogenesis inhibitors are overexpressed

ANP and BNP were strongly upregulated in the ischemic myocardium, as measured by

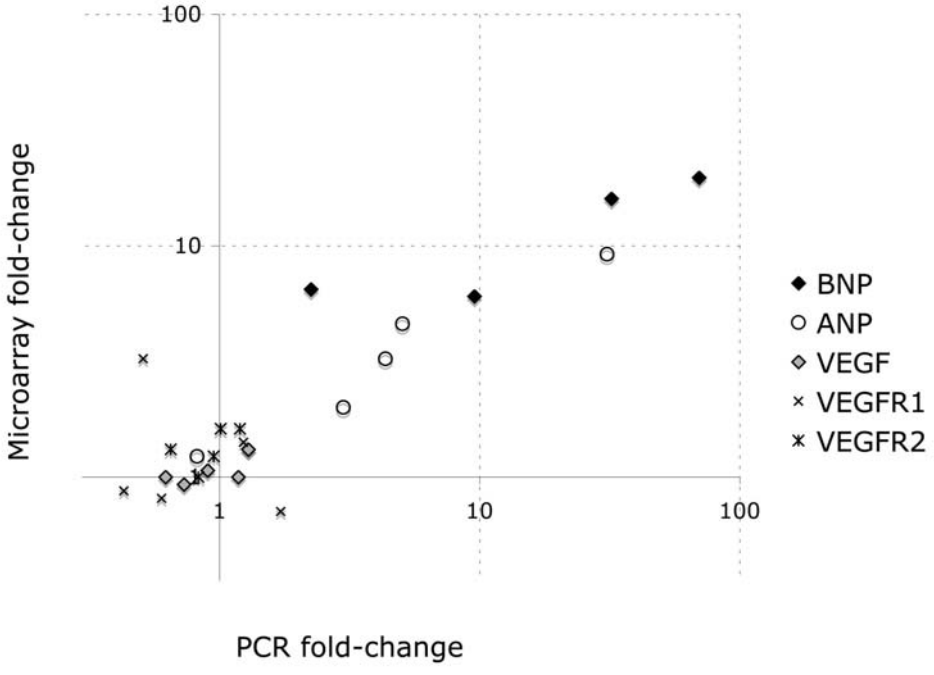

Fig. 20. Gene expression by PCR and microarray (study IV).
Note the logarithmic scale. Strong correlation between PCR and microarray values for ANP (r^2=0.91) and BNP (r^2=0.87). VEGF, VEGFR1 and VEGFR2 had a fold-change around one in most measurements.
Microarray values were lower than the PCR values in the higher ranges of fold-change.

PCR in study IV and by microarray in studies IV and V. ANP and BNP have mostly been described in the setting of heart failure. None of our patients had clinical heart failure, low ejection fraction or treatment with angiotensin converting enzyme inhibitors. Furthermore there was no perfusion defect at rest SPECT and histology ruled out fibrosis and inflammation. Therefore we think that the ANP and BNP expression increase was caused by the intermittent ischemia itself.
Recently a link between ischemia and ANP/BNP expression has been shown since HIF-1α partly regulates natriuretic peptide expression [147]. Natriuretic peptides also inhibit VEGF transcription and signalling [38, 148], thereby acting as an angiogenesis inhibitor.
SERPINE2 [149] and IGFBP3 [40], both upregulated in the ischemic area in study V, also have inhibitory properties on angiogenesis.
These factors may be important angiogenesis inhibitors in stable angina pectoris.
Further research is warranted to establish the cause-effect relationship and time course.

7.3.3 Other potential angiogenesis activators

Several of the differentially regulated genes (study V, tables 2 and 3) turned out to have a described pro-angiogenic role directly or indirectly. Examples are Connective Tissue Growth Factor [150], Autotaxin [151], Versican [152], Biglycan [153] and the Thrombin receptor [154]. Their pro-angiogenic properties have mostly been observed by in vitro-models. They may have important functions in angiogenesis associated with myocardial ischemia and need to be studied further in that context.

7.3.4 Anti-apoptosis and muscle-related genes

Several of the overexpressed angiogenesis activators (study V, table 2) also have anti-apoptotic properties. Immediate Early Response 3 (IER3) was also overexpressed and has anti-apoptotic functions [155], but no described role in angiogenesis.
Four overexpressed genes were muscle related. Interestingly, Tropomyosin 3 (TPM3) has been associated with increased contractility [156].
The increased expression of anti-apoptotic and muscle-related genes might partially explain the preserved left ventricular function after chronic coronary total occlusion.

7.3.5 Inter-related gene pathways

The complexity of the matter is increased as the differentially regulated genes not only have effects on angiogenesis and apoptosis by themselves, but also up- or downregulate each other. Some of these documented relationships are shown graphically in figure 21. Interestingly at least seven of the differentially expressed genes are regulated by TGF beta. The exact role of TGF beta in ischemia and angiogenesis is not yet known, although it is known to regulate many peptide growth factors.

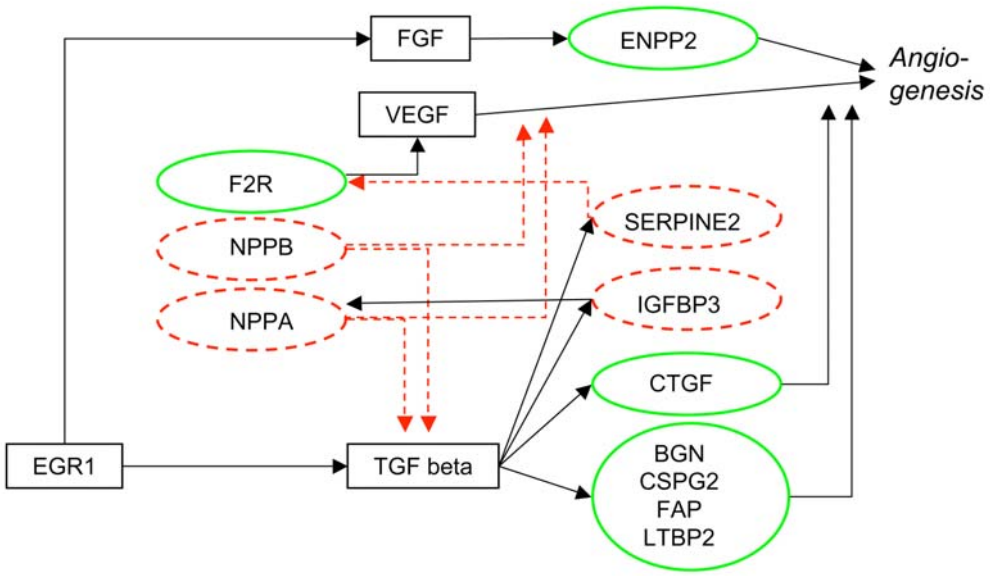

Fig. 21. Relationships between some differentially expressed genes. Differentially expressed genes are shown in ovals. Green ovals show genes with pro-angiogenic function, red dotted ovals genes with anti-angiogenic function. Black boxes signify important regulatory genes, not consistently differentially expressed in our material. Black arrows show positive regulation, red dotted arrows show inhibition.
FGF is Fibroblast Growth Factor, VEGF is Vascular Endothelial Growth Factor, EGR1 is early growth response 1 and TGF beta is Transforming Growth Factor beta. Other gene abbreviations as in Table 5.

7.3.6 Multiple testing – false positives and false negatives

Microarray studies generate a huge amount of data as the expression of thousands of genes is measured. Traditional statistical methods for hypothesis testing are appropriate when there are few hypotheses and many observations. Here the situation is the opposite, with 12158 hypotheses (genes) and 8 pairs of observations (study V). In study IV, the problem of multiple testing was partially circumvented by prospectively defining a list of 20 candidate genes.

The null hypothesis would be that the expression of one particular gene is unchanged. But how much change is defined as a significant change? Most studies use fold-change cut-off values of 1.7 to 2.0. Lower expression changes may sometimes also be biologically relevant, i.e. when several genes in the same pathway are upregulated.

The results of microarray studies are usually summarized in lists of differentially regulated genes. Typically only a few percent of the genes in a microarray study will be differentially regulated (5.4% in study V). In other words, the pre-test probability of differential regulation of one particular gene is low. If we assume that our method has 99% sensitivity and specificity and that 5% of genes are differentially regulated, 16% of the genes in the list will be false positives.

There are several ways to select differentially regulated genes.

One way is to use a simple t-test and take the genes with the lowest p-values. The p-value threshold for significance should be corrected for multiple testing. The most common correction is the Bonferroni method. If we use the familiar $p=0.05$ for one hypothesis, then $p=0.05/12158=0.000004$ would be the equivalent adjusted p-value for

12158 hypotheses [157]. This would however give a very short and conservative list, with many false negatives. In many experiments, not even one gene would have a p-value under 0.000004. On the other hand, if we use the unadjusted p=0.05, 5% or 608 genes will be positive by chance alone. Another approach is to simply rank the genes by p-value and take the lowest 1% or so, without a significance threshold.
Several biostatistical methods have been developed to overcome this problem, and the lists of differentially expressed genes will look quite different depending on which criteria were used. [158].
The *False Discovery Rate (FDR)* method calculates a value for the false-positive probability for each gene by permutation methods [94]. Depending on the type of experiment, a conservative low FDR (i.e. 0.10) may be chosen, or in more hypothesis-generating studies a more relaxed FDR (i.e. 0.40) might be appropriate. A lower FDR (type one error) gives a shorter gene list and a higher miss-rate (false-negatives or type two error) and vice versa.
We chose to use a pragmatic algorithm where 5 of 8 subjects should have change call "increase" and the mean fold change should be at least 1.7 (or 5 of 8 change call "decrease" and mean fold change below 1/1.7=0.588). This way we use the internal statistics of Affymetrix microarrays [159]. Each gene is there represented not on one but rather on 2 x 16 spots. Therefore a statistical significance value for expression change can be calculated for each gene. If the p-value is below the threshold (default 0.0025) change call is set to "increase" or "decrease", otherwise "no change". This calculation is separate from the fold-change calculation, which is made by another algorithm. Within the obtained gene list we calculated the FDR value and the t-test p-value as additional information.
Regardless which statistics are used for microarray data, key expression changes will need validation by more precise real-time PCR. This was done for five genes in study IV and we found a good correlation between PCR and microarray data (figure 20). ANP and BNP had large expression changes, and although the amount of expression change was underestimated by microarray, both methods ranked amount of expression change in the same order. The correlation was also very high (ANP r^2=0.91 and BNP r^2=0.87). The other three genes (VEGF, VEGF-R1, VEGF-R2) were identified with fold-change around one in all subjects by both methods, and therefore the correlation coefficient was not meaningful.
Further possible analysis methods include significance testing of differential regulation of groups of genes or pathways instead of individual genes. The available pre-defined pathways or gene groups were however not yet relevant to myocardial ischemia and angiogenesis. We expect a rapid development in this field.
In conclusion, any list of differentially expressed genes from microarray data will inevitably contain false-positives, and some true-positives will be missed. We are therefore planning more real-time PCR measurements to confirm key findings from microarrays and also to check some "usual suspects" which we might have missed.

8 MR. A 5 YEARS LATER

Five years have passed. Mr. A. (see section 2) still suffers from angina pectoris. He was included in the EUROINJECT ONE trial (study II) and received active treatment with plasmid VEGF injections. He improved from symptom class 3 to 2. His coronary angiogram was unchanged. SPECT imaging showed less ischemia in the treated area.

After the study was unblinded, he is told about the results and the prognosis of his disease. We return to the questions in section 2.

Will gene therapy help him?
Plasmid VEGF improves perfusion and function in the injected area.

Will his symptoms improve?
Yes, they probably will. Even with placebo, there is a good chance to improve within the next year. Plasmid VEGF had a tendency to decrease nitroglycerin use more than placebo.

Is he at high risk of dying?
Not really. The mortality is about 3% annually, much lower than after myocardial infarction or in heart failure.

Which genes are active in his ischemic area?
VEGF and most other well-known angiogenesis stimulators are not overexpressed, but several other novel angiogenic and anti-apoptotic factors are overexpressed.

Why does his recurrent ischemia not cure him from angina pectoris by inducing collateral arteries?
This is the million dollar question. It could be related to the high expression of several angiogenesis inhibitors like ANP and BNP in ischemic myocardium in stable angina pectoris.

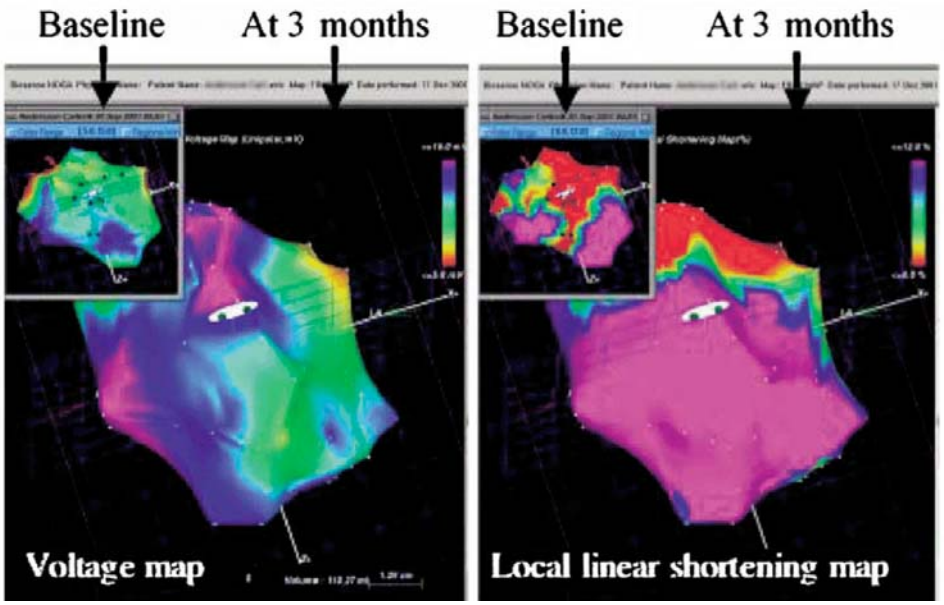

Fig. 22. NOGA electromechanical mapping of the left ventricle in Mr. A.
Small inserted maps are baseline, black dots are the injection sites. Large maps show follow-up mapping. Voltage map (left): no major change. Local linear shortening map (right): improved systolic function in the injected area.

9 GENERAL CONCLUSIONS

a. Intramyocardial plasmid encoding VEGF (phVEGF-A_{165}) was safe. Percutaneous delivery was safer than via thoracotomy, but still caused several catheter-related complications (study I and II). The biological effect was proven by improvement in perfusion and wall motion in the phVEGF-A_{165}-treated area compared to placebo (study II and [96]). Study II was not powered to detect symptomatic effects, however there was a tendency to lower nitroglycerin use after phVEGF-A_{165} compared to placebo. Symptom class and exercise capacity did not differ between active and placebo groups. The symptomatic effect needs to be tested in a larger trial.

b. Refractory angina pectoris has a rather low mortality, slightly higher than stable angina pectoris, but much lower than after myocardial infarction. Spontaneous symptomatic improvement is common and new revascularisation options arise in a subset of patients within a year (study III).

c. Most well-known angiogenesis stimulators are not overexpressed in reversibly ischemic myocardium in stable angina pectoris. In contrast, several genes with angiogenesis inhibiting, anti-apoptotic and muscle-related function were upregulated. This might contribute to the premature plateau of collateral growth observed in stable angina pectoris and also to the preserved left ventricular function even after a total coronary occlusion (study IV and V).

9.1 FUTURE DIRECTIONS

Patients with impaired left ventricular function due to hibernation might be good candidates for phVEGF-A_{165} treatment since the most robust effect in our trials was on local wall motion.
Technical advances in imaging should also allow a more precise measurement of myocardial perfusion changes after angiogenic gene therapy.
Are the patients with symptomatic improvement the same ones that have improved on SPECT and exercise tests? This could be elucidated by further analysis of data from study II.
The role and effect of exercise training in chronic angina pectoris should also be further explored. Exercise could also be used together with gene therapy.
The gene expression data on angiogenesis inhibitors could be expanded by looking at other patient categories. Is the gene expression pattern different in diabetics, where we know that angiogenesis is impaired?
It would be interesting to study the gene expression in human hibernating myocardium. The gene expression in individual cells or vessels could be studied with laser microdissection technique.

10 ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all at the Departments of Cardiology, Clinical Physiology and Thoracic Surgery at Karolinska University Hospital who have helped and supported me during the work on this thesis.

In particular, I wish to thank:

Professor Christer Sylvén, my main supervisor, for inspiring me with his enthusiasm for scientific discovery and for taking me to less travelled trails in cardiology research. The route we travelled was not the shortest, but this way I saw (and learned) a lot. Thank you for rapid and stringent feed-back on all days and hours of the week and for all the time you spent on raising me in science.

Associate professor Viktor Drvota, my efficient second supervisor, for keeping me on track and deadlines and for matter-of-fact advice, appreciated by a matter-of-fact type like me.

The Department of Internal Medicine in Huddinge, for providing facilities, time and opportunities for research. Special thanks to Anna Maria Bernstein for excellent collaboration during my time as responsible for fifth term medical students.

Inger Hagerman, Eva Stråât and Cecilia Linde, former and present heads of the Department of Cardiology for encouraging research and allowing me to take time off from clinical duties.

Professor Lars Rydén, for positive support.

Co-author Thomas Gustafsson for friendly and supportive co-operation, expert advice and for keeping me at an appropriate stress level to take things from A to B.

Co-authors in the EUROINJECT trial, especially Jens Kastrup, Mariann Gyöngyösi Hans Erik Bøtker and Witold Ruzyllo.

Co-authors Göran Källner, Jacek Nowak and Magnus Söderberg for constructive criticism and good cooperation.

Eva Wärdell and Anwar Siddiqui at the Cardiology basic science lab, Novum, and Jessica Norrbom and Helene Fischer at the Clinical physiology basic science lab, for excellent help with protein and gene analyses.

Research nurses Birgitta Welin-Berger, Åsa Hernberg, Margareta Berglund and Alicia Kursonowa, for invaluable help without which this research would not be possible.

Room-mates and colleagues Loghman Henareh and Bita Sadigh Lindell for innumerable discussions on all kinds of topics from world politics to research tactics.

Colleagues at the Department of Cardiology, especially Nondita Sarkar, Agneta Månsson Broberg, Hans Berglund, Reidar Winter and Tomas Jernberg, for inspiring scientific discussions.

The cath lab staff, and especially colleagues Toomas Särev, Shams Y Hassan, Jens Jensen, Risto Jussila, Bo Lindvall, Habib Mir Akhbari and Michael Uchto, for teaching me invasive thinking.

My friends and scientific colleagues Dirk Wuttge and Ulrik Sartipy for valuable advice.

My brother Christian, sister Birgitta and parents Paul and Marie-Louise for everything.

My wife Annika and children Ingrid and Samuel for being the sun and stars of my universe and so many other things.

All patients who took part in the studies.

This thesis was supported by grants from Karolinska Institutet, the Swedish Heart and Lung Foundation and the Swedish society for medicine.

11 RELATED PUBLICATIONS CO-AUTHORED BY ANDREAS RÜCK

1. Sarkar N, Rück A, Källner G, Hassan SY, Blomberg P, Islam KB, van der Linden J, Lindblom D, Nygren AT, Lind B, Brodin LA, Drvota V, Sylvén C: **Effects of intramyocardial injection of phVEGF-A165 as sole therapy in patients with refractory coronary artery disease--12-month follow-up: angiogenic gene therapy.** *J Intern Med* 2001, **250:**373-381.

   Related to study I.

2. Gyöngyösi M, Khorsand A, Zamini S, Sperker W, Strehblow C, Kastrup J, Jørgensen E, Hesse B, Tägil K, Bøtker HE, Ruzyllo W, Teresinska A, Dudek D, Hubalewska A, Rück A, Nielsen SS, Graf S, Mundigler G, Nowak J, Sochor H, Maurer G, Glogar D, Sylvén C: **NOGA-guided analysis of regional myocardial perfusion abnormalities treated with intramyocardial injections of plasmid encoding vascular endothelial growth factor A-165 in patients with chronic myocardial ischemia: subanalysis of the EUROINJECT-ONE multicenter double-blind randomized study.** *Circulation* 2005, **112:**I157-165.

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