Human-like atherosclerosis in minipigs: a new model for detection and treatment of vulnerable plaques

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PREFACE

Advanced atherosclerosis, most often through thrombosis, leads to ischemic heart disease and ischemic stroke, the leading causes of death and disability worldwide.

Advanced atherosclerosis and imaging of atherosclerosis are the focus of this dissertation with particular emphasis on the vulnerable plaque and vulnerable plaque detection.

In this thesis, aspects of advanced atherosclerosis and the vulnerable plaque in humans are first introduced. Then the basis for the selected animal models and methods used are described. Hereafter, the aims of the dissertation are formulated.

These aims are addressed in the sections "Injury with compliant and non-compliant balloons in porcine coronary arteries" and "Göttingen minipigs" and in 5 appended papers that are summarized in the section "Summary of appended papers".

The 5 papers are:

1. Thim T, Hagensen MK, Drouet L, Sollier CBd, Bonneau M, Granada JF, Nielsen LB, Paaske WP, Bøtker HE, Falk E. Familial hypercholesterolemic downsized pig with human-like coronary atherosclerosis: A model for preclinical studies. EuroIntervention 2010.

2. Thim T, Hagensen MK, Hørlyck A, Drouet L, Paaske WP, Bøtker HE, Falk E. Oversized vein grafts develop advanced atherosclerosis in hypercholesterolemic minipigs. Manuscript submitted.

3. Thim T, Hagensen MK, Hørlyck A, Kim WY, Niemann AK, Drouet L, Paaske WP, Bøtker HE, Falk E. Wall shear stress and local plaque development in stenosed carotid arteries of hypercholesterolemic minipigs. Manuscript submitted.

4. Thim T, Hagensen MK, Wallace-Bradley D, Granada JF, Kaluza GL, Drouet L, Paaske WP, Bøtker HE, Falk E. Unreliable assessment of necrotic core by VH™ IVUS in porcine coronary artery disease. Circ Cardiovasc Imaging. 2010 May 11. [Epub ahead of print].

5. Thim T, Falk E. Spatial orientation of cross-sectional images of coronary arteries: point of view in intracoronary imaging. Manuscript in preparation.

After the "Summary of appended papers" a discussion of some topics pertinent to these studies is given followed by conclusions and future directions. A summary and information about financial support of the described studies can be found just before the reference list.

ISCHEMIC HEART DISEASE

Worldwide, ischemic heart disease is the leading cause of death and more people die from ischemic heart disease in low- and middle-income countries than in high-income countries.[1]

In ischemic heart disease, the heart suffers from ischemia (Greek: isch- restriction, hema blood), i.e., insufficient blood supply. The coronary arteries supply the heart with blood and obstruction of coronary blood flow is the most important cause of heart ischemia.[2]

The most common cause of coronary blood flow obstruction is coronary artery disease and the most common coronary artery disease, by far, is atherosclerosis with or without superimposed thrombosis. Atherosclerosis with superimposed thrombosis is called atherothrombosis.[3] Selected causes of coronary blood flow obstruction are mentioned in Table 1.

Table 1. Coronary blood flow obstruction

The Leading Causes
Atherosclerosis
Atherothrombosis
Other and more rare causes, e.g.,
Myocardial bridge
Embolism (non-plaque origin)
Coronary artery dissection

The terms coronary artery disease and ischemic heart disease are sometimes used synonymously because almost all ischemic heart disease is caused by coronary artery disease. However, coronary artery disease is present in asymptomatic individuals for many years before ischemic heart disease develops.

Principally, in heart ischemia all heart tissue types suffer but clinically myocardial ischemia is most important. Myocardial ischemia may lead to myocardial infarction. Acutely, this may lead to heart failure and/or arrhythmia. Chronically, myocardial scarring may also cause heart failure and/or arrhythmia.

Key symptoms of myocardial ischemia are chest discomfort and shortness of breath but myocardial ischemia can also be clinically silent.

The first symptom of ischemic heart disease may be sudden death in up to 20 % of cases, stable angina pectoris in 40-50 %, and acute myocardial infarction in 30-40 %.[4,5]

STABLE ANGINA PECTORIS

Symptoms of myocardial ischemia are brought on when extra heart work is demanded, e.g. by physical exertion or emotional distress. Classically, the symptoms are relieved within minutes by rest and nitroglycerin.

ACUTE CORONARY SYNDROMES

Symptoms of myocardial ischemia usually start abruptly and are not relieved by rest or nitroglycerin. Without evidence of myocardial damage, the condition is referred to as unstable angina. With evidence of myocardial damage, the condition is referred to as acute myocardial infarction.[2] Both conditions may be complicated by sudden cardiac death (Table 2).

Table 2: Acute coronary syndromes

Unstable angina
Acute myocardial infarction
Sudden cardiac death

CULPRIT LESION

The lesion responsible for clinical symptoms, such as an acute coronary syndrome or stable angina pectoris, is called the culprit lesion.

CORONARY ARTERY ATHEROSCLEROSIS

CORONARY ATHEROSCLEROSIS

Atherosclerosis is a chronic immunoinflammatory disease of the intima of medium-sized and large arteries, including the coronary arteries, driven by lipids.[6] Initially, blood lipids enter the intima from the luminal side.[7] Later, a significant contribution to intimal lipids may come from small, fragile vessels entering the intima from the adventitia.[8,9]

Atherosclerosis is multifocal.[6] A focus of atherosclerosis is generally called a lesion and more advanced lesions are often referred to as plaques.[10-13]

Although atherosclerosis primarily is an intimal disease, advanced atherosclerotic plaques are also associated with medial destruction[14] and adventitial vascularization[8] and inflammation.[15]

EXPANSIVE REMODELING, PLAQUE SIZE AND STENOSIS

When a plaque forms, the artery may undergo compensatory enlargement and thereby “make room” for both a large plaque and the lumen.[16] This process is known as expansive remodeling.

Owing to expansive remodeling, a large plaque can be present with only limited luminal narrowing or without narrowing at all (Figure 1). However, not all large plaques are associated with expansive remodeling and these will cause luminal stenoses (Figure 1). A plaque causing stenosis is the most common cause of stable angina.

With expansive remodeling in mind, assessment of the coronary lumen with angiography does not give an accurate assessment of plaques harbored in the artery wall. In vivo assessment of both plaque size and expansive remodeling is possible with both invasive[17] and non-invasive[18] imaging.

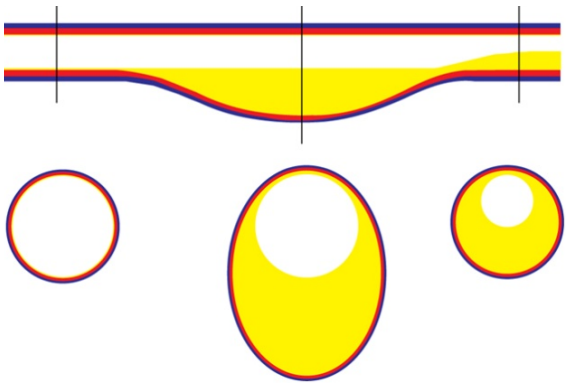

Figure 1. Atherosclerosis leads to thickening of the intima and to varying degrees of stenosis depending on degree of expansive remodeling. The normal arterial wall (left) consists of three layers: The innermost = intima (yellow), the middle = media (red), and the outermost = adventitia (blue). Atherosclerosis (middle and right) is primarily a disease of the intima and leads to thickening of the intimal layer with lesion formation. A large plaque may be present without luminal narrowing because of expansive remodeling (middle). Plaque formation may lead to luminal narrowing (stenosis) in the absence of expansive remodeling (right).

CORONARY ARTERY ATHEROTHROMBOSIS

THROMBOSED PLAQUES

A plaque with a superimposed thrombus is called a thrombosed plaque.[19]

Advances in clinical imaging technologies have paved the way for in vivo investigation of thrombosed plaques in acute coronary syndrome patients.[20,21] Thrombosed plaques have been studied for many years by pathologists, and their studies are the main source of our knowledge about thrombosed plaques. Pathologists utilize an imaging modality with higher resolution than any clinical imaging modality available today, i.e. microscopy.

THROMBOSED PLAQUE TYPES

Based on microscopic examination, thrombosed plaques can be divided into two groups, i.e. ruptured and non-ruptured plaques.

Ruptured plaques have deep injury with a defect or gap in the fibrous cap that separated its lipid-rich atheromatous core from the flowing blood.[19]

Non-ruptured plaques do not have such a deep injury with a defect or gap in their surface. The underlying mechanisms eliciting acute coronary thrombosis in non-ruptured plaques are elusive but the term plaque erosion, suggestive of a mechanism involving endothelial erosion over the plaque, is often used.[22]

Frequency of thrombosed plaque types

Overall, ruptured coronary plaques are responsible for approximately 75 % of fatal[6] and non-fatal[21] coronary thrombi. This makes the ruptured plaque the most important thrombosed plaque type.

CORONARY CONSEQUENCES OF PLAQUE RUPTURE

The fibrous cap is the tissue layer separating a lipid-rich atheromatous core from the blood. Plaque rupture is the process where the deep injury, defect, or gap in the fibrous cap arises.

Consequential to plaque rupture, lipid-rich atheromatous core material may be dislodged into the lumen and embolize to the distal coronary circulation and hemorrhage from the lumen into the plaque, i.e. plaque hemorrhage, may occur.[23,24] The contents of the lipid-rich atheromatous core are highly thrombogenic, and exposure of the lipid-rich atheromatous core leads to acute coronary thrombosis (Figure 2).[23-25]

Depending on the thrombogenic stimulus, the coronary flow, and the thrombogenicity of the blood, the thrombus may wax and wane, and lead to varying degrees of coronary flow obstruction up to total obstruction.[23-25]

A ruptured plaque with superimposed non-occluding thrombus can heal with thrombus incorporation into the plaque. This leads to plaque progression with or without significant stenosis formation.[26-29]

CLINICAL CONSEQUENCES OF PLAQUE RUPTURE

Plaque rupture followed by thrombosis is the leading cause of the acute coronary syndrome.[6,21,24]

Plaque rupture followed by thrombosis and healing may also lead to clinically silent lesion progression or progression to a lesion that causes stenosis and stable angina pectoris.[19]

THE VULNERABLE ATHEROMATOUS PLAQUE

THE VULNERABLE PLAQUE CONCEPT

The vulnerable plaque is the plaque that was present immediately before plaque thrombosis.[19] By inference from the observations of thrombosed plaques, we imagine the appearance of the plaque immediately before plaque thrombosis (Figure 3). Reliable prospective identification of vulnerable plaques is unproven.

THE VULNERABLE ATHEROMATOUS PLAQUE

The vulnerable atheromatous plaque is the ruptured plaque precursor. This plaque is also called a plaque prone to rupture or a thin-cap fibroatheroma (TCFA).[19]

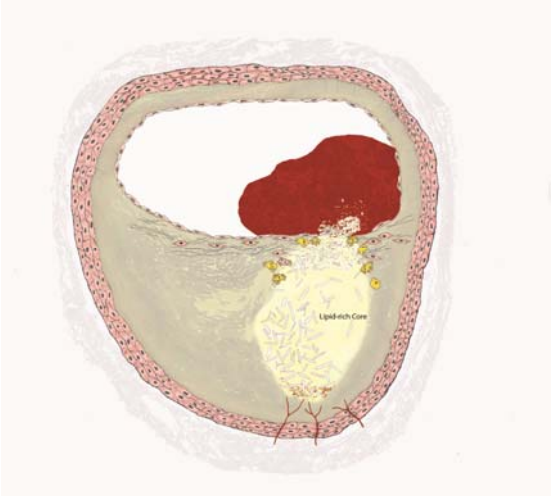

Figure 2. Ruptured plaque with superimposed thrombus causing partial luminal obstruction.
The artwork is courtesy of Mette K. Hagensen.

Since the definition of plaque rupture relies on the presence of a lipid-rich atheromatous core covered by a fibrous cap, these two structures are the key components of the vulnerable atheromatous plaque but other plaque features are also associated with vulnerable atheromatous plaques.[3] Of these, expansive remodeling, intimal microvessels, and calcification are discussed here.

THE LIPID-RICH ATHEROMATOUS CORE

A large lipid-rich atheromatous core is associated with plaque rupture and covered on average 29-34% of plaque area in ruptured human coronary plaques.[12,30,31]

The core is lipid-rich and contains free cholesterol.[32-34] The core is atheromatous (Greek: athera = gruel), i.e. of soft gruel-like substance.[6] The key feature defining the lipid-rich atheromatous core is its lack of supporting collagen.[35] Soft gruel-like and with lack of structural support, an enlarging lipid-rich atheromatous core confers mechanical instability and increasing tensile stress to the overlying fibrous cap and erodes the fibrous cap from below during enlargement.

The lipid-rich atheromatous core is acellular but it is rich in cellular debris from apoptosis and necrosis of smooth muscle cells and lipid-filled macrophages (foam cells).[10-13] Since cell death is believed to play an important role in the formation of a lipid-rich core, it is also called a necrotic core which is synonymous with lipid-rich atheromatous core.

THE FIBROUS CAP

The fibrous cap is the tissue layer separating a lipid-rich atheromatous core from the blood.[19] It consists of smooth muscle cells and the extracellular matrix they synthesize (mainly collagen and proteoglycans).[10-13] The cap also contains inflammatory cells; predominantly macrophage foam cells (Figure 4).[36]

Plaque rupture only occurs when the fibrous cap is extremely thin.[23,36] In a post mortem series of 41 ruptured coronary plaques, 95 % of the fibrous caps were < 65 µm thick (mean 23 µm).[37] Based on this finding, a thin fibrous cap is usually defined as a cap with a thickness < 65 µm.[12] Recently, a mean fibrous cap thickness of 49 µm in ruptured coronary plaques in

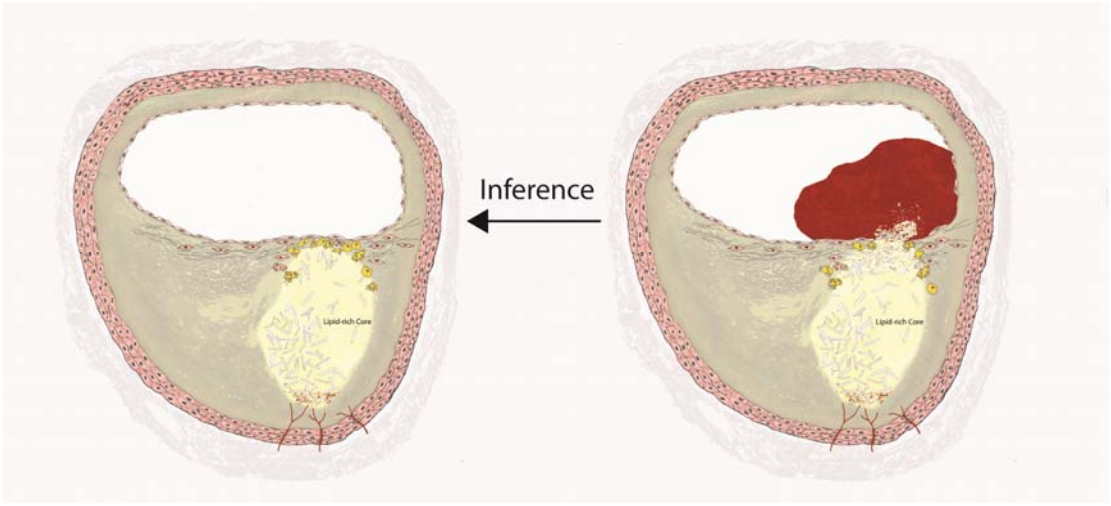

Figure 3. The appearance of the vulnerable atheromatous plaque is derived from observation of a ruptured plaque and inference.
Plaque rupture (right) with lipid-rich atheromatous core material dislodged into the lumen and superimposed thrombus. Turning back time a little removes the thrombus and heals the defect in the fibrous cap and yields the image of the plaque that was present immediately before plaque thrombosis, i.e. the vulnerable atheromatous plaque (left). The artwork is courtesy of Mette K. Hagensen.

patients with acute myocardial infarction was found with in vivo optical coherence tomography.[21]

Thinning of the fibrous cap is considered a product of increased matrix degradation by infiltrating macrophages and decreased matrix synthesis due to a decreasing number of cap smooth muscle cells.[3]

EXPANSIVE REMODELING

In acute coronary syndromes, 68 % of culprit lesions had an angiographic stenosis degree < 50 % prior to thrombus formation.[38] The main explanation for this is that stenotic plaques are relatively rare compared to non-stenotic plaques because of expansive remodelling.[16] In addition, expansive remodelling, per se, is associated with vulnerable plaques[39,40] and acute coronary syndrome.[18]

INTIMAL MICROVESSELS

The vulnerable atheromatous plaque has microvessels extending into the plaque from vasa vasorum in the adventitia (Figure 2).[41,42] Most commonly, the intimal microvessels are present at the plaque borders i.e. the base of the plaque and near shoulder regions but they may extend well into the plaque and surround the lipid-rich core.[8,43,44] The lipid-rich atheromatous core is avascular.

The microvessels are fragile and leaky as indicated by extravasation of erythrocytes and exudation of plasma proteins.[9,42,44] Bleeding from fragile microvessels within the plaque is called intraplaque hemorrhage and is associated with lipid-rich atheromatous core expansion and plaque rupture.[8,9,42,43]

CALCIFICATION

Vulnerable atheromatous plaques are less calcified than other plaque types. They contain smaller depostits of calcium that are sometimes referred to as micro-calcifications or spotty calcification.[45-47]

FATE OF VULNERABLE ATHEROMATOUS PLAQUES

Vulnerable atheromatous plaques are defined by their morphology. But it has consistently been reported that fatal myocardial infarction patients, besides their culprit lesion, usually have about two coronary vulnerable atheromatous plaques with thin fibrous caps.[23,31,37,48]

What the fate of these vulnerable atheromatous plaques would have been remains unknown. Potentially, they could have regressed, persisted, or ruptured, and the consequences of rupture could be clinically silent healing, healing leading to stenosis and stable angina pectoris, or the acute coronary syndrome.

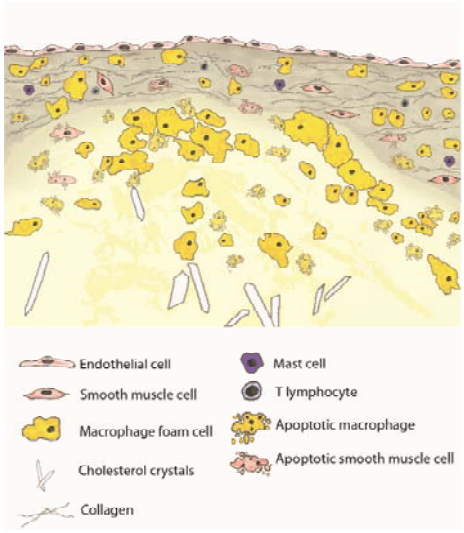

Figure 4. Thinning fibrous cap.
The cap is infiltrated by macrophages (yellow cells) and only inhabited by few apoptotic smooth muscle cells. The thickness of a thin fibrous cap corresponds to the diameter of 2-3 macrophages. The artwork is courtesy of Mette K. Hagensen.

VULNERABLE PLAQUE DETECTION

Prevention of clinical events is the ultimate goal, and perhaps local treatment of a vulnerable plaque, e.g. with a coronary stent, could prevent a clinical event. This idea is most often referred to as plaque sealing.[49]

PREMISES OF A SUCCESSFUL PLAQUE SEALING STRATEGY

The premises of this concept are: (1) Reliable assessment of plaques, e.g. reliable detection of lipid-rich atheromatous plaques with thin fibrous caps. (2) Reliable prediction of the fate of an individual lipid-rich atheromatous plaque with a thin fibrous cap. (3) Application of the local treatment reduces the risk of clinical events.

As described, the fate of an individual lipid-rich atheromatous plaque with a thin fibrous cap is still largely unknown. Additionally, it remains unproven that application of local treatment to suspected vulnerable plaques reduces the risk of clinical events.

The assessment of plaques with imaging technologies aimed at vulnerable plaque detection, so-called vulnerable plaque detectors, has improved in recent years. However, vulnerable plaque detectors deserve further investigation and improvement.

VULNERABLE PLAQUE DETECTORS

Because of expansive remodelling, lumen assessment with angiography is not well-suited for vulnerable plaque detection. Only imaging modalities that make assessment of the coronary artery wall and thereby plaque characteristics are potential vulnerable plaque detectors (Table 3). Most of these evaluate plaque morphology but some evaluate mechanical or chemical characteristics of plaques. With imaging modalities focusing on morphology, it is possible to simultaneously assess more than one characteristic, such as lipid-rich core size, core-lumen proximity (fibrous cap thickeness), total plaque area, and remodelling.[50] Likewise, palpography and elastography evaluate plaque mechanical properties that are affected by both fibrous cap and lipid-rich atheromatous core.

A key question in vulnerable plaque detector development is, of course, whether the suggested technology reliably assesses plaque characteristics as claimed. This question can be addressed in preclinical animal models where imaging results can be compared with results from post mortem microscopic examination. Such a study is described in paper 4, where the reliability of an intracoronary imaging modality focusing on plaque morphology is assessed.

Table 3. Examples of potential coronary vulnerable plaque detectors

Non-invasive
- Computed tomography[18]
- Magnetic resonance imaging[51,52]

Invasive (intracoronary imaging)
- Angioscopy[20,53]
- Thermography[54,55]
- Intravascular ultrasound (IVUS)[56]
  - Grayscale[46,57]
  - Tissue characterization[50,58]
  - Palpography/Elastography[59,60]
- Optical coherence tomography (OCT)[61]
- Near infrared (NIR) spectroscopy[62,63]
- Intravascular magnetic resonance imaging[64]

THE CAROTID ARTERY AND AORTA

The concepts described for coronary arteries in sections 2-5 also apply in the carotid arteries and aorta.

CAROTID ARTERY ATHEROTHROMBOSIS

Atherothrombosis in the carotid artery causes stroke through embolization of lipid-rich atheromatous core and thrombus material to the brain, or through obstruction of carotid artery blood flow.

Plaque rupture is found in approximately 90 % of thrombotically active carotid plaques causing stroke.[65] In the carotid artery, vulnerable atheromatous plaques are most frequently located near the carotid bifurcation.[66]

LIPID-RICH ATHEROMATOUS CORE AND FIBROUS CAP

In carotid artery plaques causing transient ischemic attacks or stroke, the lipid-rich atheromatous core covered 40 % of plaque area and the minimal fibrous cap thickness was around 80 µm.[67,68]

In ruptured aortic plaques, the lipid-rich atheromatous core covered 60 % of plaque area the minimal fibrous cap thickness was around 130 µm.[32,69]

The differences in cap thickness and lipid-rich atheromatous core between ruptured plaques in coronary arteries, carotid arteries, and aorta may reflect differences in vessel wall tension, being lowest in the coronary arteries, intermediate in carotid arteries, and highest in the aorta.

AORTOCORONARY VEIN GRAFT ATHEROTHROMBOSIS

Aortocoronary vein graft disease can be divided into three discrete, but pathophysiologically linked, processes: thrombosis, intimal hyperplasia, and atherosclerosis.[70,71]

Vein graft failure is vein graft occlusion which is usually caused by thrombosis. In early vein graft failure, thrombosis is largely related to technical factors limiting graft blood flow. Vein grafts, that do not occlude early, develop intimal hyperplasia which rarely causes significant stenosis in itself. Intimal hyperplasia may, however, form the soil in which atherosclerotic plaques can develop. Late vein graft failure is caused by rupture of an atherosclerotic plaque in the vein graft leading to thrombotic occlusion.[72] Thereby, the pathogenesis of late graft failure is similar to the pathogenesis of arterial atherothrombosis.[3]

The risk factors for atherosclerosis in aortocoronary vein grafts are also the same as for native coronary artery atherosclerosis with elevated plasma cholesterol being the most important risk factor.[70] However, vein graft atherosclerosis with atherothrombotic complications develops much more rapidly in aortocoronary vein grafts than in native coronary arteries.[73]

ANIMAL MODEL CONSIDERATIONS

This section covers some of the considerations that formed the basis for the selection of the animal models used in sections 12 and 13 as well as papers 1-4. In section 12, we wanted a model that allowed coronary interventions. In section 13 and papers 1-4, we wanted a model that allowed coronary interventions, would not grow to excessively over time, and would develop atherosclerosis.

ANIMAL MODEL SIZE

For preclinal evaluation of imaging modalities aimed at vulnerable plaque detection, it would be preferable to have an animal model close to human size. Small animals, e.g. mice and rabbits are too small for testing of intracoronary imaging modalities in the coronary arteries. At the other end of the spectrum, a farm pig weighing 200 kg is difficult to handle and will not fit into clinical scanners, such as computed tomography or magnetic resonance imaging scanners.

Minipigs are smaller pigs that, depending on the minipig strain, have varying growth rates and full-grown body weights. They can, thereby, be maintained at a body size that allows longer term studies and investigations with clinical scanners and intracoronary imaging catheters. Minipigs were therefore chosen for the longer-term studies reported in section 13 and papers 1-4. Since the study reported in section 12 was an acute study, young farm pigs were sufficient. These are more affordable than minipigs.

SUSCEPTIBILITY TO ATHEROSCLEROSIS

In section 13 and papers 1-4, we sought an animal model that would develop atherosclerosis. Plasma cholesterol is the fuel for atherogenesis but atherogenesis also depends on susceptibility to atherosclerosis (Table 4), which is genetically determined and varies between species and strains within the same species. Susceptibility to atherosclerosis is also modulated by other risk factors, such as hypertension and diabetes. Diabetes has been used to increase susceptibility in pig models.[74,75]

The combination of elevated plasma cholesterol and susceptibility to atherosclerosis lead to generalized acceleration of atherosclerosis development. Methods for acceleration of atherosclerosis at specific loci are discussed in section 10.

Table 4. Mathematics of atherosclerosis

Cholesterol level
+ Susceptibility to atherosclerosis
Atherosclerosis

SUSCEPTIBILITY TO HYPERCHOLESTEROLEMIA

In order to get atherosclerosis, high cholesterol levels are needed. In animals, spontaneous cholesterol levels are generally very low. In mice and pigs, for instance, spontaneous total cholesterol is 2-2.5 mmol/l of which a significant amount belongs to the high density lipoprotein fraction.[76-78] Two main approaches are utilized to raise cholesterol levels in animal models: (1) Supplying an atherogenic diet. (2) Using animals genetically susceptible to hypercholesterolemia (Table 5). These two approaches are often combined. The susceptibility to plasma cholesterol elevation on a certain atherogenic diet varies between species and strains.

Table 5. Mathematics of hypercholesterolemia

Diet
+ Susceptibility to hypercholesterolemia
Hypercholesterolemia

ATHEROGENIC DIETS

Atherogenic diets are diets used to promote atherogenesis through elevation of plasma cholesterol levels. Most commonly, atherogenic diets are enriched in both cholesterol (0.5-4 % of diet weight) and saturated fat (5-40 % of diet weight). When the intake of cholesterol is high, 7-α-hydroxylase, an enzyme involved in cholesterol elimination, can be upregulated in the liver. This can increase cholesterol elimination and attenuate the effect of the atherogenic diet. In mice, cholate in the diet inhibits 7-α-hydroxylase upregulation.[79] Therefore cholate is often added to atherogenic diets (0.5-2 % of diet weight).

RAPACZ PIGS

This section provides background information on the down-sized Rapacz pigs used in papers 1-4.

THE "ORIGINAL" RAPACZ PIGS

The first publication on the Rapacz pigs was in Science in 1986.[80] Rapacz and co-workers had assessed cholesterol levels on more than 14,000 farm pigs and identified farm pigs with elevated cholesterol levels. The elevated cholesterol levels were originally ascribed to mutations in lipoproteins, and based on studies on skin fibroblast low density lipoprotein receptor activity, it was decided that the pigs had normal low density lipoprotein receptor activity.[80]

These pigs, named Rapacz pigs, developed coronary atherosclerosis.[80,81] One year old pigs had macrophage foam cells in the intima. More advanced coronary atherosclerotic plaques with necrotic cores, calcification, intimal microvessels, and intraplaque hemorrhage were observed in pig more than two years old (Figure 5).[81-83]

In 1998, Rapacz and co-workers described hypercholesterolemic farm pigs with mutation in the low density lipoprotein receptor.[84] Thereby, referring to the Rapacz pigs can be equivocal in terms of the underlying genetic cause of hypercholesterolemia.

Taken together, the Rapacz pig is a highly interesting animal model because they develop advanced coronary atherosclerosis which is rare in animals. However, they have been available for more than 20 years and have hardly been used in preclinical studies because they become too big to handle weighing > 200 kg before they are two years old.

DOWN-SIZED RAPACZ PIGS IN THE UNITED STATES

In acknowledgement of the limitations related to size, Rapacz and co-workers started down-sizing the Rapacz pigs as early as 1989 by crossing them with Potbelly pigs. Down-sized Rapacz pigs are now held by the University of Wisconsin, Madison, and used by the Cardiovascular Research Foundation at The Skirball Center for Cardiovascular Research. So far, one paper with data on iliac artery atherosclerosis in two of these down-sized pigs on regular pig diet, low in fat and cholesterol, has been published.[85]

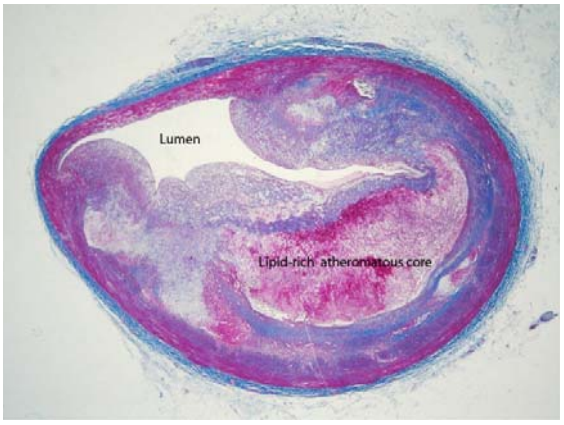

Figure 5. Cross section of coronary artery from 29 month old female Rapacz farm pig.
The coronary artery was not perfusion fixed and the lumen is collapsed. Stain: Masson's trichrome, collagen is blue and smooth muscle cells and erythrocytes are red. There is a large lipid-rich atheromatous core and hemorrhage within the plaque. The image is courtesy of Erling Falk.

DOWN-SIZED RAPACZ PIGS IN EUROPE

In 1999, Rapacz farm pigs with the low density lipoprotein receptor mutation[84,86] were imported to France. In France, Professor Ludovic Drouet and his co-workers, down-sized the Rapacz pigs first by crossing them with a medium-sized pig, the Chinese Meishan. The product of this cross was then crossed with a local minipig from Bretoncelles. During the down-sizing process, all pigs were genotyped for the low density lipoprotein receptor mutation,[84,86] and the minipigs obtained from this two step down-sizing are homozygous for the mutation.

POTENTIAL ADVERSE EFFECTS OF DOWN-SIZING

Crossing the original Rapacz with other pig strains as described, places the low density lipoprotein receptor mutation on a different genetic background. Here, the susceptibility to hypercholesterolemia may be more or less pronounced than in the original Rapacz pigs. Likewise, the susceptibility to atherosclerosis may be more or less pronounced than in the original Rapacz pigs.

Whether the genetic mutation in the down-sized pigs yields a useful phenotype, therefore, needs to be examined. This is addressed in papers 1-3.

LOCAL LESION ACCELERATION

Local lesion acceleration methods are used in "Injury with compliant and non-compliant balloons in porcine coronary arteries"and papers 1-3. This section mentions different methods as well as some reasons for and limitations to their application.

RESPONSE TO INJURY

In normocholesteroemic pigs, the response to coronary artery balloon overstretch injury inflicted with non-compliant balloons is well-described. The coronary arteries heal with neointima formation.[87] More severe injury leads to more pronounced neointima formation.[88] The acute response is hemorrhage and thrombosis, followed by inflammation and neoangiogenesis, followed again by organization with connective (scar) tissue formation.[89-91] This healing process completes within 4 weeks.[89] Many other methods have been used to induce neointima formation. Some examples are given in Table 6.

NEOINTIMA – SOIL FOR ATHEROSCLEROSIS DEVELOPMENT

It is commonly believed that atherosclerotic lesions form at locations with preexisting intima thickening and that the intima constitutes a soil in which atherosclerotic lesions develop.

Many of the methods used to induce neointima in normocholesterolemic animals have therefore been applied in hypercholesterolemic animals to induce or accelerate atherosclerotic lesion development. Beside the examples given in Table 6, many others exist, such as thermal balloon injury,[108] intraarterial wire injury,[109] perivascular electric injury,[110,111] radiation injury,[112] or combinations of more than one injury method including stenting.[113]

LOCAL LESION ACCELERATION – PROS AND CONS

Development of spontaneous atherosclerotic lesions takes time and makes animal atherosclerosis studies lengthy and costly. Acceleration of lesion development with the mentioned methods may reduce time consumption and costs. Also, local lesion acceleration methods may increase the total number of lesions available for investigation, e.g. with an imaging technique. The possibility for investigators to choose lesion location may also be preferable, e.g. in imaging studies.

In humans, atherosclerosis normally develops without the described local injuries to the artery wall, and the described local lesion acceleration models may therefore not be completely representative of spontaneous lesions. However, non-compliant balloons, stents, and vein grafts, are used to treat obstructive atherosclerosis in humans. Therefore injury models can be particularly relevant for studies on effects of and complications to these treatment modalities.

Although the development of accelerated lesions may differ from the spontaneous development, accelerated lesions can be useful in studies of specific atherosclerosis related processes,[106,107] or in imaging studies where the assessment of an imaging modality's ability to detect a certain plaque component can be assessed, such as paper 4.

Table 6. Examples of different injury methods used to induce neointima and accelerate atherosclerosis

Injury method	Neointima formation	Atherosclerosis acceleration
Non-compliant balloon	Porcine carotid and coronary[87-91]	
Compliant balloon	Rat carotid[92]	Porcine carotid[93], rabbit aorta[9]
Vein graft	Porcine[94], rabbit, mouse[95] carotid	Rabbit[96,97] and mouse[98,99] carotid
Ligation	Mouse carotid[100]	Mouse carotid[101]
Stenosing collar	Porcine[102] and rabbit[103] carotid	Porcine[104,105] and mouse[106,107] carotid

AIMS

The overall aims of the studies were to develop an animal model of advanced atherosclerosis with human-like vulnerable plaque morphology and to use this animal model to test an imaging modality aimed at vulnerable plaque detection. This was translated into the following specific aims:

To compare acute effects of coronary balloon injuries inflicted with compliant and non-compliant balloons in pigs

To investigate susceptibility to hypercholesterolemia and spontaneous coronary atherosclerosis in Göttingen minipigs

To investigate susceptibility to hypercholesterolemia and coronary spontaneous and balloon-accelerated atherosclerosis in down-sized Rapacz pigs

To investigate locally accelerated atherosclerosis in vein grafts in down-sized Rapacz pigs

To investigate locally accelerated atherosclerosis by surgically induced carotid artery stenosis in down-sized Rapacz pigs

To test the ability of VH™ IVUS to accurately identify and assess necrotic core in porcine coronary atherosclerosis

Addressing aim VI, we encountered a pivotal question. We are always careful in keeping the same know orientation of our microscopy slides, so we always view the slides from the same side. Viewing them from the opposite side would be like viewing mirror images. We were unable to find information on the orientation of IVUS images and therefore set the additional aim:

To determine the orientation of IVUS images.

The specific aims are addressed in

"Injury with compliant and non-compliant balloons in porcine coronary arteries"

"Göttingen minipigs"

Paper 1

Paper 2

Paper 3

Paper 4

Paper 5

Papers 1-5 are found in the appendices 1-5 and are summarized in "Summary of appended papers".

INJURY WITH COMPLIANT AND NON-COMPLIANT BALLOONS IN PORCINE CORONARY ARTERIES

This section includes data that are not included in the appended papers.

BACKGROUND

Coronary artery response to non-compliant balloon injury in normocholesterolemic pigs is well-described.[87-91] Increasing overstretch inflicts increasing arterial injury.[87,88] Most often, this type of balloon is used to inflict deep injury to elicit a pronounced neointimal response.

In animal experiments, compliant balloons, e.g. the Fogarty balloon catheter, are used to deendothelialize.[9] In accordance with the descriptive term, compliant balloon injury is often regarded as being tantamount to very superficial injury to the endothelium without injury to the media and adventitia.

In this experiment, acute changes resulting from injuries inflicted with compliant and non-compliant balloons in porcine coronary arteries are described.

METHODS

Female Danish farm pigs (n=6, 40 kg) were anesthetized, and coronary balloon injuries were inflicted under fluoroscopic guidance with non-compliant angioplasty (3.5-4.0x12mm) balloons and compliant (3F Fogarty balloon catheters) as specified in Table 7.

In two pigs (1,2) non-compliant balloons were used, while compliant balloons were used in four pigs (3-6). For inflations without pull back, the target balloon to artery ratio was 1.5. With non-compliant balloons, this was obtained with pressures from 12-14 atmospheres. For non-compliant balloon pull backs, balloon pressure of 1 atmosphere was used. With compliant balloons, balloon pressure was controlled by hand and not measured.

After balloon injury, Evans blue dye (1 g in 50 ml isotonic saline) was injected intravenously over 15 minutes using an infusion pump and allowed to circulate in the pigs for 1 hour before the pigs were killed with a pentobarbital overdose.

The hearts were excised and the coronary arteries were cut open longitudinally for inspection.

Evans blue dye has a molecular weight of 960.8 g/mol. It

Table 7. Coronary balloon injuries with compliant and non-compliant balloons in farm pigs

Pig	Left anterior descending	Left circumflex	Right
1	No injury	Non-compliant balloon, 1 inflation	Non-compliant balloon , 1 pull-back
2	Non-compliant balloon, 1 inflation	No injury	Non-compliant balloon , 1 pull-back
3	Compliant balloon, 1 pull-back	No injury	Compliant balloon, 2 pull-backs
4	Compliant balloon, 2 pull-backs	No injury	Compliant balloon, 1 pull-back
5	Compliant balloon, no pull-back, 1 "hard" inflation,	No injury	Compliant balloon, 2 pull-backs
6	Compliant balloon, no pull-back, 2 "hard" inflations	No injury	Compliant balloon, 2 pull-backs

binds readily to proteins in plasma, and tissue. In plasma, Evans blue dye binds mainly to albumin. Albumin has 8-14 binding sites for Evans blue and unless the concentration of Evans blue exceeds the binding capacity of albumin, very little unbound Evans blue remains in plasma. Albumin with a molecular weight of 69 kg/mol (kDalton) does not penetrate intact endothelium to any large degree, and thus albumin bound Evans blue does not likely enter the arterial wall where the endothelium is intact.[114,115]

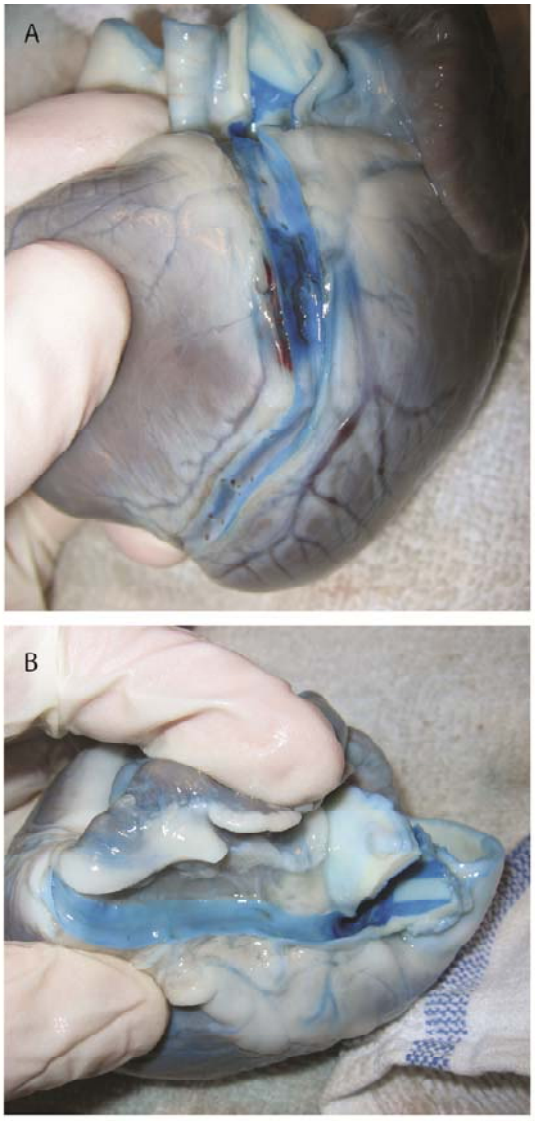

Figure 6. Coronary artery injury inflicted with non-compliant balloon.
A, single inflation overstretch injury in the left anterior descending artery with rupture of the medial layer, periarterial hemorrhage, and heavy Evans blue staining. The patchy staining proximal and distal to the balloon injury site is due to endothelial injury inflicted with the guide wire and balloon during positioning of the balloon. B, noncompliant balloon pull back in the right coronary artery did not cause rupture of the media, but the artery is clearly stained with Evans blue in the entire arterial circumference corresponding to the pull back track.

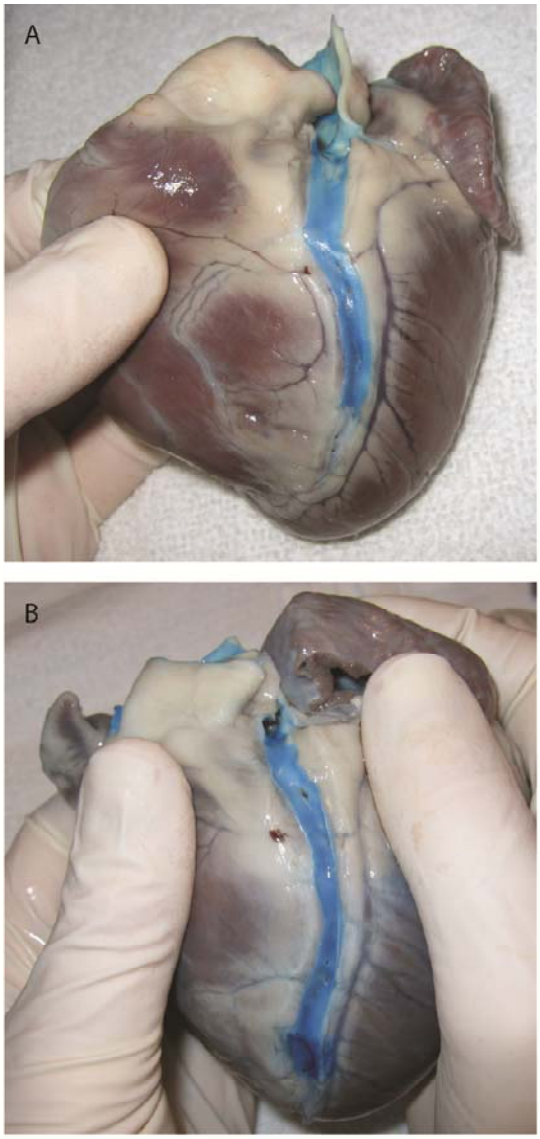

Figure 7. Left anterior descending coronary artery injury inflicted with compliant balloon.
A, Evans blue staining corresponds to the pull back track. The staining ends abruptly outside the track. B, in this pull back track there was medial rupture in the most distal part.

RESULTS

With non-compliant balloons (pigs 1,2), single inflation overstretch injury induced lesions with rupture extending through the medial layer of the coronary arteries and periarterial hemorrhage (Figure 6). The injured sites were heavily stained with Evans Blue in the entire arterial circumference and a length matching balloon length. Non-compliant balloon pull back was not associated with rupture of the media, but the arteries were heavily stained with Evans blue corresponding to the pull back track, and the staining ended abruptly outside the pull back track.

With compliant balloons (pigs 3-6), balloon pull back led to heavy Evans Blue staining corresponding to the pull back track. As for non-compliant balloon pull back, the Evans blue staining ended abruptly outside the pull back track. In compliant balloon

pull-back tracks, rupture of the media was noted distally in some, but not all, pull-back tracks (Figure 7).

Hard inflation of the compliant balloons without pull-back (pigs 5,6), induced lesions that were practically indistinguishable from the non-compliant balloon single inflation overstretch injuries (Figure 8).

Ordinary instrumentation of coronary arteries with guide wires and balloons induced endothelial injury evidenced by Evans Blue staining (Figures 6 and 9). In contrasts, coronary arteries that were not instrumented or injured (pigs 1-6) were not stained with Evans Blue (Figure 9).

While untouched coronary arteries were not at all stained with Evans Blue, the aortas stained diffusely light blue (pigs 1-6). Traces of endothelial injury caused by the guide catheters were, however, heavily stained with Evans Blue and clearly visible on this light blue background (Figure 10).

Visual evaluation of the balloon injury sites suggested that overstrectch injury sites were more heavily stained than sites exposed to one or two balloon pull-backs (pigs 1,2,5,6) and that sites exposed to two pull-backs seemed more heavily stained than sites exposed to one (pigs 3,4).

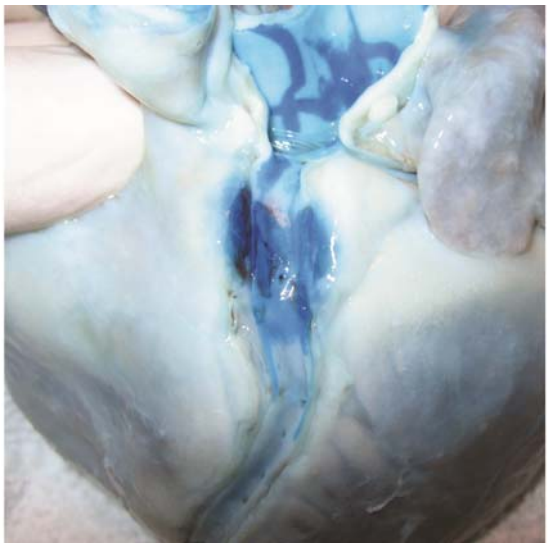

Figure 8. Left anterior descending coronary artery overstretch injury inflicted with compliant balloon.
At the injured site rupture of the medial layer, periarterial hemorrhage, and heavy Evans blue staining is visible. The appearance is very similar to the balloon injury in Figure 6.

DISCUSSION

These experiments demonstrate that overstretch injury can be induced with both compliant and non-compliant balloons. Likewise, endothelial injury without medial rupture can be induced with both compliant and non-compliant balloons.

The use of compliant balloons is often regarded as being tantamount to endothelial injury without medial injury. These experiments demonstrate that medial injury can be induced with compliant balloons, both deliberately and accidentally. This was, in fact, already acknowledged by Fogarty et al. when the catheter was introduced for extraction of arterial emboli and thrombi in 1963.[116] In our hands, injury degree seemed easier to control with non-compliant balloons compared with compliant balloons.

We observed Evans blue staining in the aorta and the coronary ostia as a result of guide catheter induced endothelial injury, and in the coronary arteries as a result of guide wire and balloon induced endothelial injury outside the intended lesion area. Since endothelial injury is associated with atherosclerosis development, these findings are thought provoking. Similar endothelial injury must be expected to occur in patients during catherization, but the significance of this remains elusive.

In rabbit aortas, Evans blue staining decreased gradually over time in balloon injured areas but Evans blue stained areas remained 6 months after the balloon injury.[115] In porcine coronary arteries, Evans blue staining was found at stented and balloon-injured sites after 12 weeks.[117] These reports, unfortunately, do not discuss Evans blue staining resulting from guide catheter or guide wire induced endothelial injury.

CONCLUSION

Very similar or identical balloon injuries could be inflicted with compliant and non-compliant balloons. The type and extent of injury depended on how the two types of balloons were used.

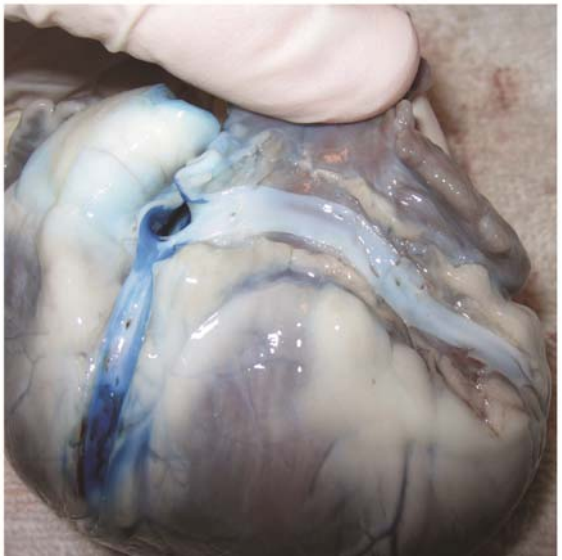

Figure 9. The left circumflex coronary artery was not instrumented and no Evans blue staining is seen.
In contrast the instrumented left anterior descending artery is clearly stained.

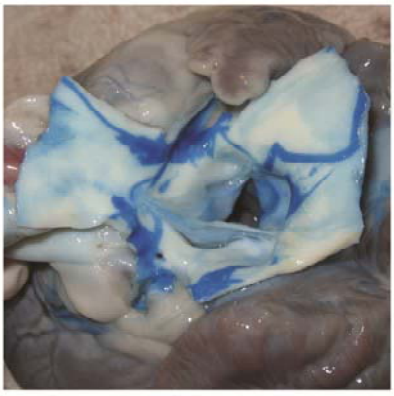

Figure 10. The aortic root cut open down to the coronary ostia.
Endothelial injury caused by the guide catheter visualized by the Evans blue staining. There are clearly stained tracks in the aorta and in the coronary ostia.

GÖTTINGEN MINIPIGS

This section includes data that are not included in the appended papers.

BACKGROUND

The Göttingen minipig is one of many strains of minipigs available for research. The Göttingen minipig, stems from the University of Göttingen, and results from breeding on the Minnesota minipig, the Vietnamese Potbelly pig, and the German Landrace pig. An exclusive licence to breed the Göttingen minipig is now held by Ellegaard Göttingen Minipigs A/S in Denmark. In collaboration with Marshall BioResources, Ellegaard also delivers the Göttingen minipig to researchers in the United States and Canada.

The Göttingen minipig has been used previously in atherosclerosis research.[118-122] In these studies, the atherogenic diet contained egg yolk and cholesterol yielding a relative high cholesterol content. The cholesterol levels were only mildly elevated and with these cholesterol levels, the minipigs did develop atherosclerotic lesions, but these did not resemble advanced human plaques.[119-122]

The aim with this experiment was to investigate susceptibility to hypercholesterolemia and spontaneous coronary atherosclerosis in Göttingen minipigs fed a highly atherogenic diet containing 2 % cholesterol, 20 % saturated fat, and 1.5 % cholate.

METHODS

We acquired 19 Göttingen minipigs from Ellegaard Göttingen Minipigs A/S. The minipigs had been fed a diet added varying amounts of cholesterol (1.0-1.9 %), coconut and corn oil (22 %), and fructose (16-37 %) for 20 months from they were 3 months old. The minipigs were fed this diet in order to produce a metabolic syndrome model (Figure 11).

Between this experiment and ours, the minipigs were fed a normal pig diet. Such a diet contains very little cholesterol and fat.

After arrival at our stable facilities, the minipigs were fed an atherogenic diet (TestDiet, 583V: 2 % cholesterol, 20 % fat, and 1.5 % cholate; all percentages are percent of diet weight).

Blood samples were drawn before the minipigs were fed the atherogenic diet and after two and seven weeks on the atherogenic diet (Figure 12).

Blood samples were analyzed for alkaline phosphatase, alanine transaminase, triglycerides, total cholesterol, and high density lipoprotein cholesterol. Low density lipoprotein cholesterol was calculated with the Friedewald formula.[123]

After this the minipigs were put on a normal diet for 5 months before post mortem examination. The hearts and aorta were immersion fixed, and sections for microscopy were taken from the proximal 3 cm of the left anterior descending, left circumflex, and right coronary arteries, and from the most elevated lesion in aorta. Sections were stained with hematoxylin and eosin.

We could not measure concentrations of triglycerides lower than 0.11 mmol/l, total cholesterol lower than 1.3 mmol/l, and high density lipoprotein cholesterol lower than 0.5 mmol/l. Low density lipoprotein cholesterol concentration calculation is based on concentrations of triglycerides and total cholesterol, and when these could not be determined low density lipoprotein concentration could not be calculated. When summarizing the data, the lowest detectable value was used for values below detection level. This leads to overestimation of the mean, but it leads to less overestimation than exclusion of the values would do. For low density lipoprotein, missing values were excluded, which leads to overestimation of the means.

RESULTS

Prior to any atherogenic diet feeding, the mean total cholesterol was 1.8 mmol/l. On high fat diet, the plasma cholesterol rose to ~ 15 mmol/l after 5 months on atherogenic diet and then gradually declined to ~ 5 mmol/l. Upon reduction of diet cholesterol content to 1 %, plasma cholesterol declined even further to ~ 3 mmol/l (Figure 11). Results of plasma analyses from our experiment are presented in (Figure 12).

Originally, there were 20 minipigs in the metabolic syndrome study, but one minipig died prematurely. This minipig had coronary atherosclerosis with calcifications and lipid accumulation, including some cholesterol crystals. This finding stimulated our further studies with these pigs.

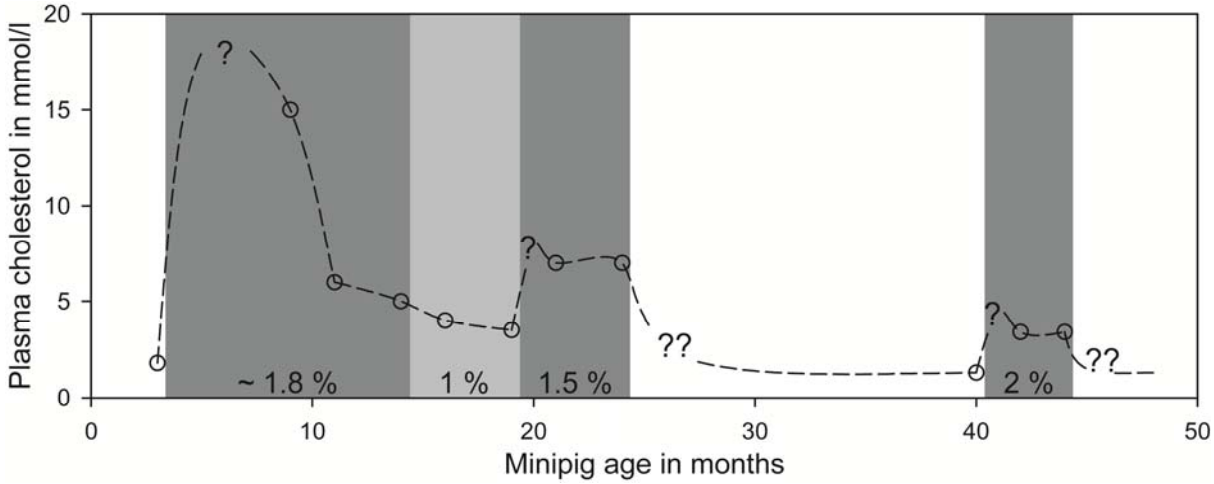

Figure 11. Time course of diet cholesterol content and plasma cholesterol levels in Göttingen minipigs.
The minipigs were fed atherogenic diets (gray background) with varying cholesterol contents. The cholesterol contents in percent weight of diet weight are given above the time axis. White background corresponds to periods where diets were not added cholesterol. The plasma cholesterol peak values and time point corresponding to these peaks are not known but do probably not coincide with the time of blood sampling (?). Neither is it known how plasma cholesterol levels declined upon withdrawal of the cholesterol-enriched diets (??).

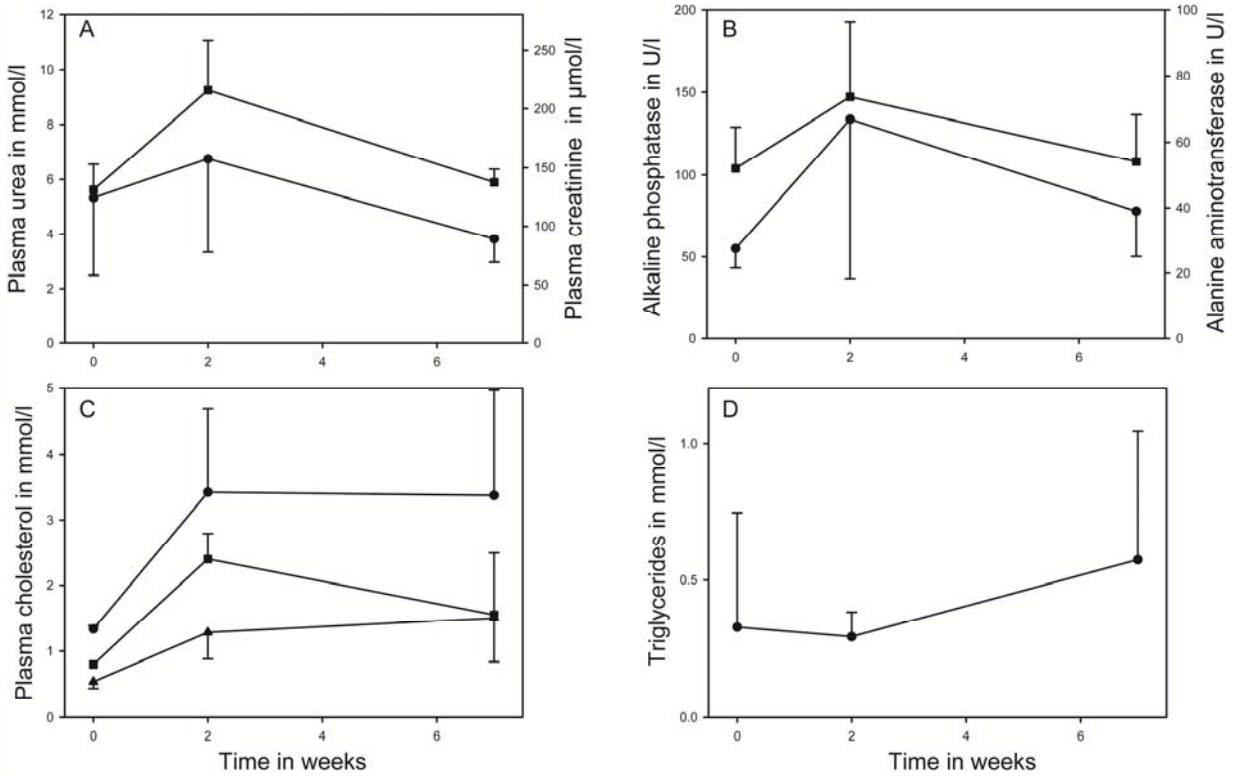

Figure 12. Time course of plasma values during atherogenic diet feeding in Göttingen minipigs.
A, plasma urea (circles) and creatinine (squares). B, plasma alkaline phophatase (circles) and alanine aminotransferase (squares). C, plasma total (circles), low density lipoprotein (squares), and high density lipoprotein (triangles) cholesterol. D, plasma triglycerides.

When we received the pigs, total cholesterol levels were around detection level, ~ 1.3 mmol/l. Cholesterol levels inclined only modestly to ~ 3.4 mmol/l after 2 and 7 weeks on our atherogenic diet. After the atherogenic diet was discontinued, no blood samples were analyzed.

Post-mortem examination of coronary arteries and aorta revealed intimal thickening in all minipigs and intimal calcifications in more than half of the minipigs. Cholesterol crystals in collagenrich matrix were observed occasionally, but pools of extracellular lipid or lipid-rich atheromatous cores were not observed (Figure 13).

DISCUSSION

The design of this study is no textbook example. A sudden appearance of an opportunity was caught by eager investigators soon disappointed by their results. Before termination, the minipigs also served in an imaging study not described here. Despite the limitations imposed by the design of this experiment, some relevant observations can still be made.

There was a gradual fall in plasma cholesterol over time on the metabolic syndrome diet. Part of the explanation is likely hepatic 7-α-hydroxylase upregulation,[79] but contributions may come from a number of other homeostatic mechanisms. This phenomenon has been observed in Göttingen minipigs before and is also observed in other pig strains.[118,124]

Plasma cholesterol levels normalized after discontinuation of a diet that increased plasma cholesterol in agreement with previous reports.[119,124] If the one minipig that died prematurely were representative of the others, the decline in plasma cholesterol was associated with lesion regression, also in agreement with previous reports.[119,124]

Plasma cholesterol levels could be raised moderately in these Göttingen minipigs, but cholesterol levels were not very high for longer periods of time in this study.

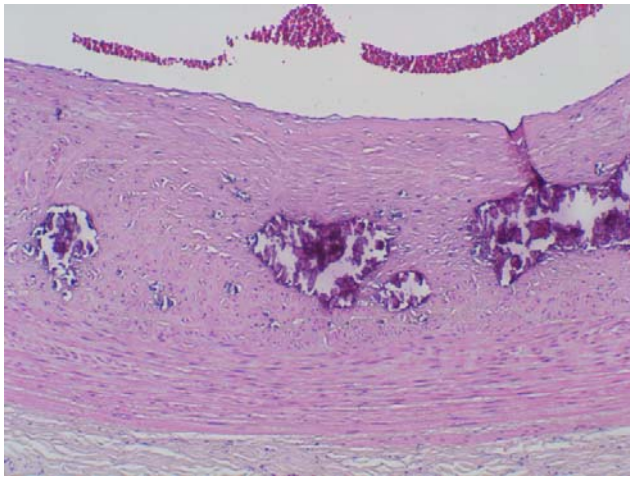

Figure 13. Atherosclerotic lesion from the left anterior descending artery of a four year old Göttingen minipig.
The intima is thickened and contains dense calicifications (Ca). There are no foam cells or extracellular lipid.

Since these minipigs were not subjected to longer periods of severe hypercholesterolemia and they were subjected to periods with very low cholesterol levels, it is hard to make firm conclusions about their susceptibility to atherosclerosis. Their susceptibility was not sufficient to lead to advanced atherosclerosis with the presented plasma cholesterol levels and time periods. Accordingly, previous reports have not demonstrated advanced coronary atherosclerosis in Göttingen minipigs.[118-122,124]

CONCLUSION

Plasma cholesterol levels were only modestly elevated in the Göttingen minipigs fed an atherogenic diet. Susceptibility to hypercholesterolemia was not pronounced and susceptibility to atherosclerosis could not be adequately ascertained.

SUMMARY OF APPENDED PAPERS

PAPER 1:

Adult down-sized Rapacz (9 months old) were fed an atherogenic diet for 4 months and subjected to coronary artery balloon injury.

The atherogenic diet caused pronounced hypercholesterolemia and the minipigs had advanced atherosclerotic plaques with lipid-rich atheromatous cores in the coronary arteries both within and outside the balloon injured sites (Figure 14).

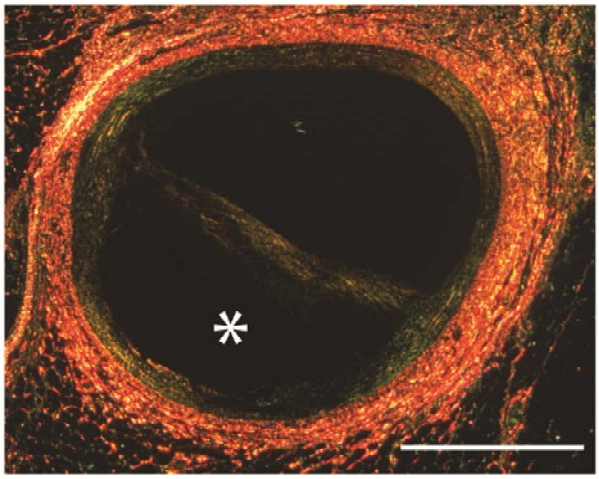

Figure 14. Spontaneously developed coronary atheromatous plaque.
Picrosirius red stain viewed under polarized light demonstrates lack of collagen in the lipid-rich atheromatous (necrotic) core marked with asterisk. The scale bar is 1mm.

PAPER 2:

In the same down-sized Rapacz pigs, autologous reversed jugular vein grafts inserted end-to-end into the transected common carotid artery of down-sized Rapacz pigs, plaques with lipid-rich atheromatous cores were found only when graft diameter exceeded carotid artery diameter (Figure 15). This finding indicates increased vein graft diameter, probably through altered shear stress, as a risk factor for plaque development in vein grafts.

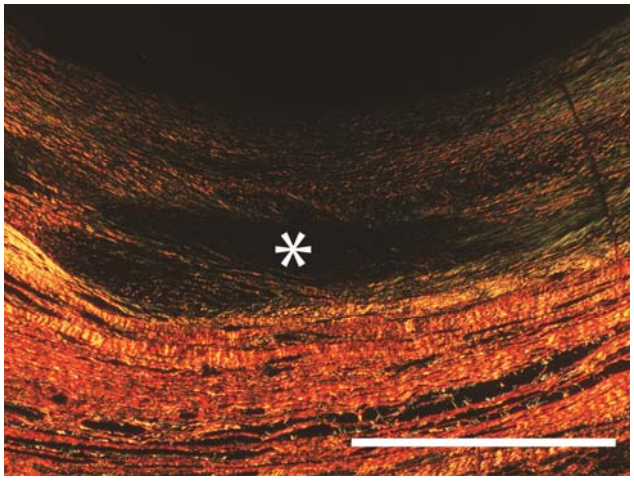

Figure 15. Vein graft plaque.
Picrosirius red stain viewed under polarized light demonstrates lack of collagen in the lipid-rich atheromatous (necrotic) core marked with asterisk. The scale bar is 1mm.

PAPER 3:

In the same down-sized Rapacz pigs, common carotid blood flow changes were induced with a perivascular collar. Wall shear stress was described with computational fluid dynamics based on assessment of carotid artery blood flow and geometry with magnetic resonance imaging (Figure 16). Plaque development was associated with thrombotic occlusion of the stenosed segment or with low and oscillatory shear stress in the post-stenotic segment.

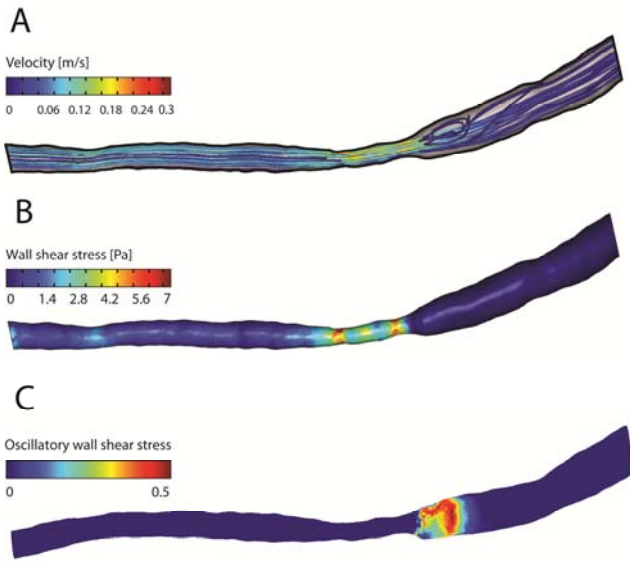

Figure 16. Carotid geometry, flow and wall shear stress described with magnetic resonance imaging and computational fluid dynamics.
A, flow velocity. B, wall shear stress. C, oscillatory wall shear stress.

PAPER 4:

VH™ IVUS is a technology aimed at tissue characterization of plaque component, also called plaque or tissue mapping. VH IVUS generates tissue composition color-coded maps with 4 color codes: red for necrotic core, light green for fibrofatty tissue, dark green for fibrous tissue, and white for dense calcium. In this study, we assessed how well the red areas in VH IVUS corresponded with necrotic areas in histology and found that VH IVUS did not reliably predict size or location of necrotic areas (Figure 17).

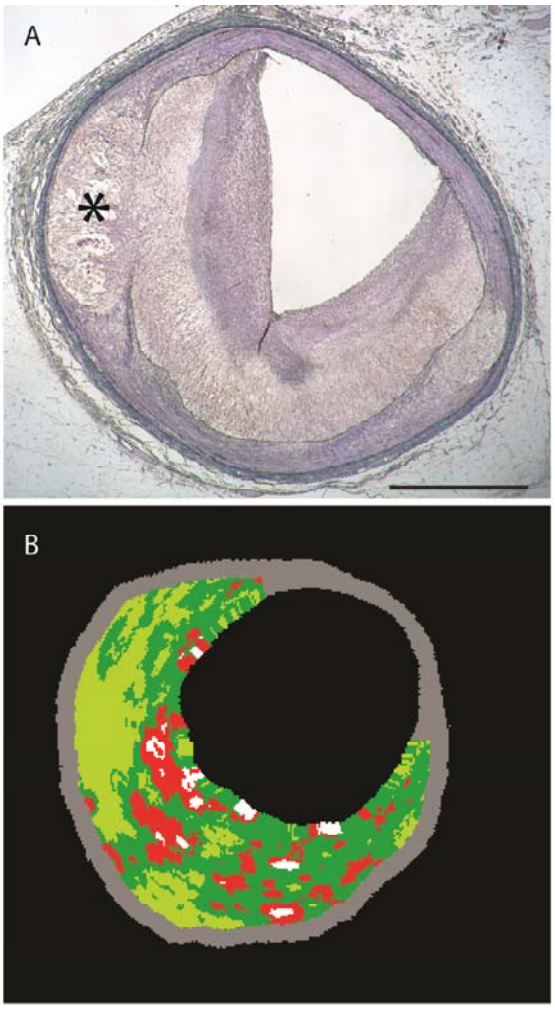

Figure 17. Spontaneously developed plaque with necrotic core.
A: Trichrome-elastin stain (collagen blue, elastin black, smooth muscle and blood cells red). A large necrotic core is indicated by asterisk. B: VH IVUS display: the necrotic core is light green (fibrofatty tissue) rather than red (necrotic core). The scale bar is 1 mm.

PAPER 5:

One should imaginge looking at IVUS images from a point of view proximal to the displayed cross-section. It is therefore essential that one also views the microscopy slides from a point of view proximal to the cross section (Figure 18). These considerations have implications for the development and evaluation of imaging technologies and also apply when IVUS images are compared to images obtained with other imaging modalities such as optical coherence tomography, magnetic resonance imaging and computed tomography.

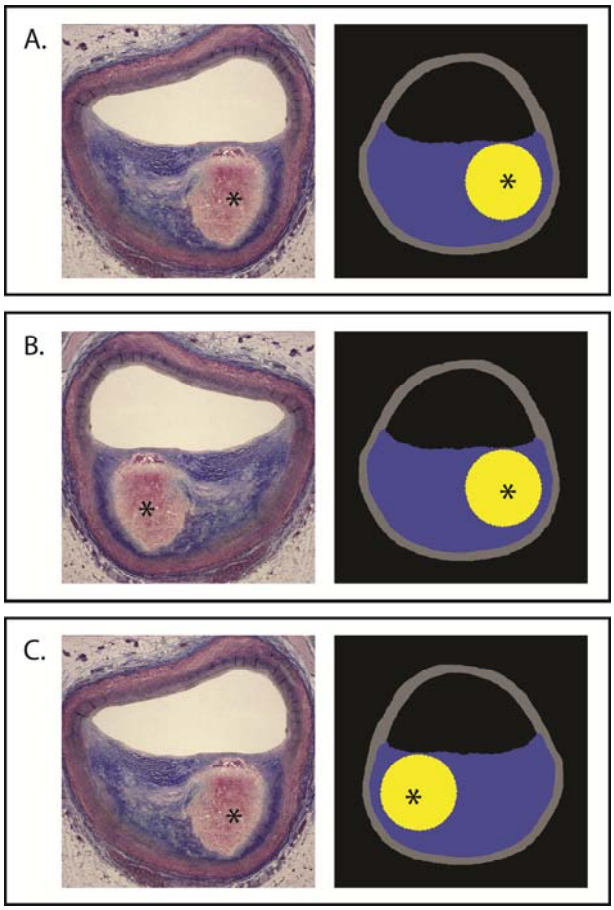

Figure 18. Comparison of images from microscopy and intravascular imaging.
The software displays the necrotic core (asterisk) in yellow and fibrous tissue in blue. In A, the size and intraplaque location of the necrotic core match with microscopy. In B and C, this is not the case. If the orientation of the images is the same, then firm conclusions on the technology can be made. Without known point of view, it is difficult to say if the method or the technology is flawed.

DISCUSSION

Today, coronary interventional tools such as coronary stents are predominantly tested in coronary arteries of healthy young pigs,[125] while they are mainly used to treat advanced atherosclerotic plaques in coronary arteries of human adults. Also, some coronary imaging tools are developed and marketed without preclinical validation in animal models of coronary atherosclerosis.[126,127] Preferentially, these technologies should be tested in an experimental coronary atherosclerosis model. However, the experience with the Göttingen minipigs described in section 13 illustrates how a vast amount of money and time can be invested in the development of such a model with a relatively disappointing return. This probably explains why coronary atherosclerosis models are rarely used in preclinical testing.

Ideally, a model should be easy to handle, affordable, and reproducible. That is, the animals should not be too large and lesions should develop within reasonable time in most or all animals.

Paper 1 describes the development of a model of advanced coronary atherosclerotic lesions in adult minipigs. The model includes generalized atherosclerosis acceleration via monogenetically increased susceptibility to hypercholesterolemia combined with atherogenic diet to exacerbate hypercholesterolemia. Added to this, we used coronary balloon injury for local lesion acceleration via local increase in atherosclerosis susceptibility. Combining these methods, an acceptable frequency of coronary atherosclerotic plaques was observed. Importantly, the observed plaques contained all components of human coronary plaques, including the dangerous lipid-rich atheromatous core. Hence, the criteria for a useful model were met.

In paper 4, we interrogated coronary atherosclerotic lesions in this model with an intracoronary imaging modality, VH IVUS.[126,127] The technology is currently used as endpoint in clinical trials,[128] it is commercially available, and some may even make prognostic inferences on its basis. We assessed the technology's ability to assess the most important and dangerous plaque component, the lipid-rich atheromatous core. We found that the technology did not reliably detect or quantify this plaque component. This is in agreement with a previous study interrogating porcine coronary lesions induced by injecting liposomes into the coronary wall.[129] This previous study received criticism mainly on two points: that the technology was not developed for use in accelerated lesions and that the technology was not developed for porcine use.[130] These points can really not be contested, and they also point to the main limitations of paper 4. However, in addition to balloon-accelerated lesions, we also interrogated spontaneously developed plaques in paper 4.

VH IVUS was validated ex vivo on human cadaver coronary atherosclerotic lesions.[126,127] The ex vivo imaging was done at room temperature, with saline in the coronary arteries, and without cardiac motion.[126,127] In contrast, in vivo imaging is done at body temperature, with blood in the coronary arteries, and with cardiac motion. Thereby, imaging conditions differ between in vivo and ex vivo, and in vivo validation attempts in animal models could preferably supplement ex vivo validation in human cadaver material.

Notably, VH IVUS is used in clinical studies to interrogate stents and other coronary diseases than atherosclerosis for which it has never been validated.[131,132] To interpret such studies, more experience with the technology over a broader range of lesions is desirable. As pointed out earlier, even with the limitations dictated by an animal model, it is puzzling if homogenous lesions are systematically misclassified.[133]

A discussion of similarities and differences between the coronary artery plaques observed in the reported minipig model and human coronary artery plaques is given below to address the critique of the porcine coronary atherosclerosis model in imaging modality validation studies.

HUMAN ATHEROSCLEROSIS IS HETEROGENEOUS

Human atherosclerosis is heterogenous.[10-13]

Atherosclerotic plaque components can be divided into four: fibrous tissue, necrosis (lipid-rich atheromatous core), inflammation, and calcification. The relative contribution of these components to total plaque area varies between different plaques. As a crude overall average, the relative contributions in the major epicardial coronary arteries of acute myocardial infarction or sudden cardiac death victims are: fibrous tissue 68 %, necrosis 16 %, inflammation 8 %, and calcification 8 %.[134]

The relative contributions of plaque components and their intraplaque locations constitute plaque morphology, which is the basis of current classification schemes of atherosclerotic lesions (Table 8).[10-13]

Lesions of the same type share key characteristics, e.g. all atheromatous plaques have a necrotic core. However, they still may look very different based on the relative contribution of the components to total plaque area and their intraplaque locations.

Additionally, each of the plaque components is defined by key characteristics but display heterogeneity both within the same plaque but also between different plaques.

A widely used characteristic for defining lipid-rich atheromatous (necrotic) core is its lack of supporting

Table 8. Modified American Heart Association classification based on morphological description[10-13]

Intimal lesion type	Description
Normal intima/adaptive intimal thickening	Normal connective tissue containing smooth muscle cells. No lipid accumulation or macrophages.
Intimal xanthoma/fatty streak	Normal intima except foam cell accumulation near lumen
Non-atheromatous plaque	Extracellular accumulation of lipid and connective tissue with fibrosis with or without calcification. No atheromatous lipid-rich (necrotic) core.
Atheromatous plaque	Contains a lipid-rich atheromatous (necrotic) core.

collagen.[3,6,26,27,35,38] The lipid-rich atheromatous necrotic core contains cellular debris and extracellular lipids including cholesterol crystals. These components are closely associated with necrotic cores but are not necessarily present in the entire area covered by a necrotic core. Also, these components may be present outside necrotic cores. Likewise, necrosis may or may not be associated with calcification,[135,136] and calcification can be present outside necrotic cores.

Likewise, fibrous tissue of atherosclerotic lesions has varying collagen density and lipid content, and calcifications may be large and dense or in the form of microcalcifications.

"HUMAN-LIKE" LESIONS

Considering the heterogeneity of human atherosclerosis, deciding whether atherosclerosis in a given animal model reflects human atherosclerosis is complicated. Should the model display a certain type of plaque, or should the animal model display the heterogeneity to be representative of human disease? In papers 1-4, the main focus was on advanced plaques with morphology similar to human vulnerable plaque morphology or at least with all the components of these plaques. Some questions related to how representative the observed lesions are to human disease are suggested in Table 9.

Spontaneous or injury-induced intimal thickening can be observed in normocholesterolemic animals. These lesions only contain the fibrous component of atherosclerosis and the models are, generally, not considered models of atherosclerosis.

Foam cell accumulation in intima, either spontaneously formed or injury-induced, is generally accepted as models of early atherosclerotic lesions.

Advanced atherosclerotic lesions, i.e. plaques, and in particular the atheromatous plaque with a lipid-rich atheromatous necrotic core, are observed in hypercholesterolemic mice and pigs, such as the Rapacz.[80-83,106,107]

Also in the down-sized Rapacz, lesions with all the relevant plaque components were found. The lipid-rich atheromatous cores of these plaques could be identified with the same methods used in the evaluation of human plaque. Also, the relative contribution and intraplaque location of plaque components resembled that of human advanced plaque. Thereby, the described model seems useful for studies pertinent to advanced atherosclerotic lesions, e.g. studies on detection and treatment of vulnerable plaques.

As in any animal model, there are limitations. These are mainly related to the study of accelerated lesions and the use of an animal model per se. It has been reported that atherosclerosis differs slightly in morphology in humans with monogenic causes of hypercholesterolemia compared to those without a monogenic cause.[137] Additionally, locally accelerated lesions may not be entirely representative for spontaneous disease. These points are particularly relevant in studies focusing on lesion development. In studies focusing on imaging modality testing or intervention, it is more pertinent to consider whether the relevant plaque components are present and whether they resemble the human components, rather than how they developed. Porcine plaque components may differ somewhat from human plaque components in composition. In the microscope, a relatively lower occurrence of cholesterol crystal in porcine atheromatous lipid-rich cores compared to human cores seems to be the most striking difference.[138]

Table 9. "Human-like" lesions?

Are relevant plaque components present?
Do the plaque components resemble human plaque components?
Is the plaque morphology similar to human plaque morphology?

VULNERABLE PLAQUES IN ANIMAL MODELS

A generally accepted animal model of plaque rupture does not exist.[139-141] Since spontaneous plaque rupture is only rarely observed in animal models, the term vulnerable plaque is ill-defined in animal models.

To study plaque rupture in animal models, investigators can wait for spontaneous plaque rupture. However, this may occur silently without alerting the investigator to its presence, so some investigators induce plaque rupture mechanically. Although these models can be excellent for studies on the consequences of plaque rupture[107], they do not unveil the causes and events leading to spontaneous rupture.

Although, the concept of vulnerable plaques is ill-defined in animal models, the occurrence of plaques with morphology similar or nearly identical to human vulnerable plaques is useful in a study like the one in paper 4.

CAROTID ARTERIES AND VEIN GRAFTS

The focus of this discussion has been similarities and differences between the coronary artery plaques observed in the reported minipig model and humans. Although the origin has been the coronary arteries, the same considerations apply for the vein graft and carotid artery plaques described in papers 2 and 3. Lipid-rich atheromatous core is as important in the carotid artery and aortocoronary vein grafts and therefore the occurrence of this plaque characteristic in the described models is encouraging. Papers 2 and 3 discuss the influence of flow and wall shear stress on local plaque development in two different atherosclerosis acceleration models.

CONCLUSIONS

SPECIFIC AIMS

Very similar or identical balloon injuries could be inflicted with compliant and non-compliant balloons. The type and extent of injury depended on how the two types of balloons were used.

Plasma cholesterol levels were only modestly elevated in the Göttingen minipigs fed an atherogenic diet. Susceptibility to hypercholesterolemia was not pronounced and susceptibility to atherosclerosis could not be adequately ascertained.

The down-sized Rapacz pigs were susceptible to hypercholesterolemia and spontaneous and balloon-accelerated coronary atherosclerosis.

Vein graft atherosclerosis was observed in the down-sized Rapacz pigs, and vein graft atherosclerosis seemed linked to vein graft diameter exceeding that of the grafted artery.

Surgically induced carotid artery stenosis in down-sized Rapacz pigs was associated with plaque development when the stenoses caused thrombotic occlusion of the stenosed segment or

caused low and oscillatory shear stress in the post-stenotic segment.

VH IVUS™ did not reliably identify and assess necrotic core in porcine coronary artery disease.

One should imaginge looking at IVUS images from a point of view proximal to the displayed cross-section. It is therefore essential that one also views the microscopy slides from a point of view proximal to the cross section for comparisons.

OVERALL AIMS

We developed an animal model of advanced atherosclerosis with human-like vulnerable plaque morphology. The usefulness of this animal model was demonstrated in a study testing an imaging modality aimed at vulnerable plaque detection in humans.

FUTURE DIRECTIONS

In a large animal model of advanced coronary artery atherosclerotic plaques a number of interesting studies could be performed.

IMAGING STUDIES

In parallel with the study presented in paper 4, many other imaging modalities could be tested with comparison of imaging results with histology. To increase the benefit of an experimental study, more than one imaging modality could be tested in the same study, e.g. non-invasive imaging with computed tomography, magnetic resonance imaging, or ultrasound could be followed by interrogation with one or two intravascular imaging techniques before postmortem microscopic examination. In such studies both coronary (paper 1) and carotid models (papers 2 and 3) could be useful. Also, testing of new contrast agents or serial imaging to monitor atherosclerosis progression is possible.

DEVICE TESTING

Intracoronary device, e.g. drug eluting stent, testing will likely continue to be based primarily on healthy young pigs. Results from this model, e.g. reduction of restenosis, are regarded as reproducible and representative of the response in human coronary arteries. And the model is much more affordable than atherosclerotic minipigs. Longer-term safety studies, e.g. looking at factors related to late stent thrombosis, cannot be performed in normal farm pigs that will outgrow the implanted stents. Such studies need to be performed in minipigs.

Special problems related to arterial healing around stent struts placed in the necrotic core, however, can only be tested in animals with coronary arteries containing plaques with necrotic cores such as the model presented in paper 1.

METHODOLOGY

The experience gained in the papers 1-3 is helpful in future studies where acceleration of atherosclerosis is desired. The described methods can be applied in new studies with the same minipig strain or to other hypercholesterolemic minipig strains taking parameters such as vein graft diameter into consideration in the study design.

ATHEROGENESIS

Studying atherogenesis and effects of different strategies that impact atherogenesis are easier and cheaper in smaller models, e.g. mice. However, with open eyes and mind, one may incidentally observe interesting aspects of atherogenesis and atherosclerosis, e.g. in studies designed to test imaging and interventional tools.

SUMMARY

Advanced atherosclerosis, through thrombosis, leads to ischemic heart disease and ischemic stroke, the leading causes of death and disability worldwide. Advanced atherosclerosis and imaging of atherosclerosis are the focus of this dissertation with particular emphasis on the vulnerable plaque and vulnerable plaque detection.

Aspects of advanced atherosclerosis and the vulnerable plaque in humans are described along with the basis for the selected minipig models and methods for atherosclerosis acceleration used.

The overall aims of the studies were to develop an animal model of advanced atherosclerosis with human-like vulnerable plaque morphology and use this animal model to test an imaging modality aimed at vulnerable plaque detection.

The first aim is addressed in 3 papers, where accelerated plaque development in the coronary and carotid arteries is investigated in down-sized Rapacz pigs. Down-sized Rapacz pigs are minipigs with familial hypercholesterolemia caused by a mutation in the low density lipoprotein receptor.

Paper 1 describes the lipid profile in the down-sized Rapacz on chow and atherogenic diets and spontaneously developed and balloon-accelerated coronary plaque with a morphology that resembles the morphology of human vulnerable plaque.

Paper 2 describes vein graft disease in internal jugular vein grafts inserted into the common carotid artery. Plaques with necrotic cores were found in oversized vein grafts only indicating an effect of flow and shear stress on plaque development.

Paper 3 describes the effects of wall shear stress on local plaque development in surgically stenosed common carotid arteries in the down-sized Rapacz pigs. This study indicated that the combination of low and oscillatory wall shear stress was needed for development of advanced plaque.

In paper 4, we interrogated coronary lesions in the down-sized Rapacz with a commercially available diagnostic tool VH IVUS. It is claimed that VH IVUS can characterize the tissue components that constitute plaque reliably. However, we found that VH IVUS does not reliably assess the most important plaque component of all, i.e. the necrotic core.

In conclusion, we developed an animal model of advanced atherosclerosis with human-like vulnerable plaque morphology. The usefulness of this animal model was demonstrated in a study testing an imaging modality aimed at vulnerable plaque detection in humans.

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