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DEVELOPMENT OF SUPERSELECTIVE ENDOVASCULAR TISSUE ACCESS AND SAMPLING

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DEVELOPMENT OF SUPERSELECTIVE ENDOVASCULAR TISSUE ACCESS AND SAMPLING
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Till pappa.

We can only see a short distance ahead,
but we can see plenty there that needs to be done.

Alan Turing

POPULAR SCIENCE SUMMARY OF THE THESIS

Endovascular techniques involve navigation of instruments inside the blood vessels to treat or diagnose disease, with x-rays and other imaging techniques used for guidance. There are many examples of effective treatments using endovascular techniques, which are generally safer than comparable surgical methods. Endovascular treatments include opening blocked vessels in the heart and limbs, removal of blood clots in the brain and lungs, stopping internal bleeding by blocking damaged vessels, and repairing vessels such as the aorta from the inside. While endovascular treatments have been proven safe and effective, often more so than surgical interventions, they are mainly limited to treatment of diseases that occur inside the blood vessels. It would be highly useful to develop endovascular techniques that allow highly targeted access to the tissue itself, for treatment and the diagnosis of disease.

Most diseases are defined by processes that impair or change the function of one or more bodily organs (e.g. the heart), producing effects on other organs and the body as a whole, as well as symptoms of disease (e.g. chest pain, breathlessness). When a patient reports symptoms that makes the physician suspect that a certain organ is impaired, this is first investigated by means of non-invasive methods, such as blood tests or imaging. If the suspected organ is found to be affected, it can often be treated by making a preliminary diagnosis that assumes the most likely underlying cause (a preliminary diagnosis). However, in many cases, this is not sufficient, and a more specific diagnosis is needed. Particularly, if more invasive and dangerous treatments could be needed, like surgery or chemotherapy, it is vital to ensure that the diagnosis is as correct and specific as possible before treatment is initiated, so that the treatment is correct and that its risks are managed. In such cases, the next diagnostic tool used is often biopsy. A biopsy procedure involves removing a small part of the disease-affected organ to examine it using various methods such as microscopy, gene expression analysis, or protein measurements. These methods give more direct evidence of what disease process is affecting the organ; for example, cancer, an infection, or an autoimmune process. Using this evidence, the most specific and effective treatment can then be used.

Since biopsy involves slightly damaging the organ to remove a small part of it for analysis, the procedure has inherent risks. These risks vary according to what organ is biopsied and the state of the patients' general health. Some biopsy methods require surgery and general anesthesia to reach the target organ. Sometimes only a part of the organ is affected, and extraordinary accuracy is needed during biopsy to actually capture the part of the organ where the disease is located, as is often the case in cancer. Biopsy on internal organs is typically performed by puncturing the skin over the organ with a relatively large needle and quickly advancing a spring-loaded tissue collecting device to harvest a small amount of tissue. The needle is then retracted. This technique works well for organs such as the liver, the kidneys, and the superficial parts of the lungs, but it can be very difficult or technically impossible to reach smaller organs or tumors in them. Moreover, to access the brain, a hole must be drilled in the skull to create a path for the biopsy device, which increases complexity

and risks. It is therefore necessary to develop safer and more accurate biopsy methods, especially for organs that are difficult to reach safely, like the heart, central lungs, pancreas, and brain.

This thesis explores new methods to access the heart and other organs, using endovascular techniques, to treat (e.g. by transplanting cells) or perform a biopsy of the organ. Few endovascular methods of cell transplantation and biopsy exist in clinical practice. This may be due to the relatively high cost of endovascular intervention, and to the often difficult process of reaching the area of interest for injection of treatments or biopsy. Biopsy of the heart is the most commonly performed endovascular biopsy method. However, it is still relatively rarely used, partly due to concerns for complications including damage to the heart. Another reason is that heart biopsy in its current form quite often does not give useful diagnostic information. It could be that the tissue sample is not from the right part of the heart, and even if this is achieved, the analysis method has limitations. Heart biopsy is mostly analyzed by visual inspection of the tissue in a microscope, which limits detection to diseases which change the appearance of the tissue and cells. Recent developments in diagnostic methods that involve large scale automated measurement of the molecular content of a biopsy sample, such as all genes expressed or thousands of proteins found in the tissue, yield much more comprehensive and detailed information about the state of the cells in a tissue sample. This may make it possible to diagnose heart disease more efficiently and precisely when using biopsy. There are also other methods of endovascular biopsy, such as experimental harvesting of the endothelial cells which line the vessel walls. This method is not clinically established but could be a useful way of gathering and analyzing endothelial cells in patients with vascular diseases.

This thesis is based on four studies with the overall goal of developing new methods of access to and biopsy of hard-to-reach organs using endovascular navigation. First, we used an established the instrument of endovascular treatment for stroke to collect endothelial cells in a swine model, and showed that these cells can be collected and grown in culture to create a cell population. These cells could then be analyzed to investigate how diseases affect the vessel wall cells of, for example, the brain. We showed that high quality gene expression data can be generated from these cells. This technique could easily be implemented in clinical research.

Next, we improved upon an endovascular technique, the trans-vessel wall technique, which makes it possible to have a very small catheter exit the blood vessel from the inside in order to gain direct access to an organ that is difficult to reach by other means, such as the brain or pancreas. Once the catheter has entered the organ, it constitutes a working channel, allowing cells to be transplanted, or medications to be directly injected. It may even be possible to develop a biopsy device which uses the trans-vessel wall technique to take small tissue samples. We explored the use of the trans-vessel wall technique in the heart, the kidneys, and pancreas of animals for the first time, and demonstrated its safety and ease-of-use.

Finally, we created a new type of heart biopsy device, which is smaller, more flexible, and possibly safer than currently available devices. We showed that it is safe to use in healthy animals as well as those affected by heart disease, and that the small tissue samples gathered by the device can be analyzed using modern gene expression techniques to detect changes in the heart muscle caused by heart infarction. This opens the possibility to use the new heart biopsy device to gather diagnostic information in patients with failing hearts where the cause of disease is not clear, and where conventional heart biopsy may be considered too risky.

ABSTRACT

Endovascular techniques have revolutionized healthcare by allowing effective treatments of vascular diseases not possible through other surgical means. They have also largely supplanted some surgical treatments due to increased safety and equal or sometimes increased efficacy. However, most endovascular techniques concern the treatment of intravascular disease, as the ability to reach the extravascular tissue has been limited. The trans-vessel wall technique is a recent endovascular technique, in the pre-clinical stage, that allows endovascular instruments to exit the blood vessel and gain direct access to the tissue of an organ. The trans-vessel wall technique and other minimally invasive approaches could be used to treat organs directly, and to perform biopsy.

Tissue and cell biopsy are crucial parts of medical diagnosis, and the reference standard for most pathological conditions. In addition, biopsy is an important source of biological material of disease for pre-clinical research. Biopsy is performed using a variety of techniques with different levels of invasiveness, ranging from drilling through the skull to direct needle puncture with or without image guidance. There is a clear trend towards less invasive methods, which may offer a lower risk of complications and lesser need for post-biopsy monitoring, so that tissue crucial for diagnosis can be procured more quickly, safely, and preferably in outpatient settings. This thesis aimed to develop novel methods of accessing hard to reach organs using endovascular navigation, for the purposes of delivery of therapeutic substrates, or for performing biopsy.

In *Study I* we employed mechanical thrombectomy, an established endovascular treatment method for ischemic stroke, to harvest endothelial cells from vessels affected by thrombosis. We showed that the endothelial cells can be isolated from the devices and thrombus, enriched by cell culture, and analyzed using single cell RNA sequencing.

In *Study II* we tested an improved version of the trans-vessel wall technique, a method of direct tissue access using endovascular navigation, to access the heart, the kidneys, and pancreas parenchymae without causing significant hemorrhage. We showed that the myocardial wall can be accessed epicardially and endocardially, and that the kidney capsule can be accessed selectively. These access points are suitable targets for cell transplantation. The pancreas and kidney were also highly accessible for potential new biopsy techniques.

In *Study III* we developed a novel endomyocardial biopsy device that is smaller, less traumatic, and more flexible than currently available methods, which may improve lesion targeting and reduce complications. We showed that the samples gathered by the device could be reliably analyzed using RNA sequencing.

In *Study IV* we employed the novel endomyocardial biopsy technique in swine affected by myocardial infarction, showing that it is safe to use in a diseased heart and that RNA sequencing analysis of the biopsy samples could detect tissue gene expression changes caused by the myocardial infarction.

In conclusion, this thesis demonstrates a variety of novel approaches to access tissues for administration of cells and therapeutic substances, and to obtain biopsies in safer and less traumatic ways than currently possible, using modern low profile endovascular techniques. It also shows that RNA sequencing can be a valuable tool to gather as much data as possible from the small cell and tissue samples gathered using these techniques.

LIST OF SCIENTIFIC PAPERS

(* = equal contribution)

I. Jaff N*, **Grankvist R***, Muhl L, Chireh A, Sandell M, Jonsson S, Arnberg F, Eriksson U, Holmin S.

Transcriptomic analysis of the harvested endothelial cells in a swine model of mechanical thrombectomy.

Neuroradiology. 2018 Jul;60(7):759-768.

II. **Grankvist R**, Jensen-Urstad M, Clarke J, Lehtinen M, Little P, Lundberg J, Arnberg F, Jonsson S, Chien KR, Holmin S.

Superselective endovascular tissue access using trans-vessel wall technique: feasibility study for treatment applications in heart, pancreas and kidney in swine.

Journal of Internal Medicine. 2019 Apr;285(4):398-406.

III. **Grankvist R***, Chireh A*, Sandell M, Mukarram AK, Jaff N, Berggren I, Persson H, Linde C, Arnberg F, Lundberg J, Ugander M, La Manno G, Jonsson S, Daub CO, Holmin S.

Myocardial micro-biopsy procedure for molecular characterization with increased precision and reduced trauma.

Scientific Reports. 2020 May 15;10(1):8029.

IV. Chireh A*, **Grankvist R***, Sandell M, Mukarram AK, Arnberg F, Lundberg J, Daub CO, Holmin S.

Micro-biopsy for detection of gene expression changes in ischemic swine myocardium: A pilot study.

PLoS One. 2021 Apr 28;16(4):e0250582.

SCIENTIFIC PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Little P, Kvist O, **Grankvist R**, Jonsson S, Damberg P, Söderman M, Arnberg F, Holmin S.

Preserved Collateral Blood Flow in the Endovascular M2CAO Model Allows for Clinically Relevant Profiling of Injury Progression in Acute Ischemic Stroke.

PLoS One. 2017 Jan 9;12(1):e0169541.

II. Lundberg J, Jussing E, Liu Z, Meng Q, Rao M, Samén E, **Grankvist R**, Damberg P, Dodoo E, Maeurer M, Holmin S.

Safety of Intra-Arterial Injection With Tumor-Activated T Cells to the Rabbit Brain Evaluated by MRI and SPECT/CT.

Cell Transplantation. 2017 Feb 16;26(2):283-292

III. Lundberg J, **Grankvist R**, Holmin S.

The creation of an endovascular exit through the vessel wall using a minimally invasive working channel in order to reach all human organs.

Journal of Internal Medicine. 2019 Sep;286(3):309-316.

IV. Chireh A, Sandell M, **Grankvist R**, Lövljung V, Al-Saadi J, Arnberg F, Lundberg J, Settergren M, Holmin S.

Safety evaluation of high-risk myocardial micro-biopsy in a swine model.

Heart and Vessels. 2021 Nov 23.

CONTENTS

LIST OF ABBREVIATIONS

AMI	Acute myocardial infarction
CAR	Chimeric antigen receptor
CD146	Cluster of differentiation 146
CD31	Cluster of differentiation 31
cDNA	Complementary deoxyribonucleic acid
CT	Computed tomography
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
EGMV2	Endothelial cell growth medium 2
EMB	Endomyocardial biopsy
F	French catheter scale (1F = 0.33 mm)
FACS	Fluorescence activated cell sorting
LAD	Left anterior descending artery
LV	Left ventricle
micro-EMB	Micro-endomyocardial biopsy
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RV	Right ventricle
vWf	von Willebrand factor

1 INTRODUCTION

A core tenet of medicine is *primum non nocere*: first do no harm[1]. In order to help a patient, there must be trust in the institution of clinical medicine, which means that the patient must feel reassured that any treatment attempted by their doctor is first and foremost helpful. In reality, most diagnostic tests and treatments incur some sort of risk of harm to the patient. No medication is completely without side effects, and no surgery has a zero risk of complications. The balance of risk-to-benefit must be in the patient's favor, and diagnostic tests and treatments must be likely to improve the patient's health every time they are used. In this spirit, there has been a continuous trend in modern medicine to improve treatments and diagnostic tests, not only to make them more effective, but also to make them safer while remaining just as effective. Many invasive diagnostic tests have been replaced by non-invasive techniques, such as imaging, and many invasive surgical treatments have been replaced by image-guided minimally invasive techniques, such as endoscopy and laparoscopic surgery[2,3].

One powerful modality of minimally invasive diagnostics and treatment is the endovascular approach; that is, the introduction of catheters and instruments inside the blood vessels, allowing navigation to organs of interest for the performance of diagnostic and therapeutic maneuvers, such as angiography and angioplasty[4]. Endovascular techniques became prevalent towards the middle of the 20th century with the advent of catheter angiography, and have developed rapidly since, seeing the introduction of a variety of treatment methods, primarily in cardiovascular medicine and oncology. Some endovascular techniques have since been replaced by less invasive diagnostic methods, particularly large vessel angiography, which is now largely supplanted by the comparatively non-invasive modalities of computed tomography (CT) and magnetic resonance imaging (MRI) angiography. Diagnostic endovascular techniques are mainly limited to angiography in specific situations, such as cerebral angiography for vascular malformations and coronary angiography for coronary artery disease, and endovascular biopsy of the heart, liver, and intravascular tumors. Nevertheless, the endovascular route is theoretically highly useful for specific, superselective, non-surgical access to parts of organs that are otherwise hard to reach, since all organs are supplied by arteries and drained by veins, which can be used for navigation. Historically, the main limitation has been a lack of suitable technical materials in terms of catheters and devices flexible enough to safely and easily reach and treat or biopsy all parts of these organs.

2 LITERATURE REVIEW

2.1 ENDOVASCULAR TECHNIQUE

The earliest examples of endovascular techniques were using plastic tubing (catheters) placed in various vascular beds to measure hemodynamic pressure[5,6] (e.g. aorta, cardiac chambers, pulmonary artery) and to perform angiography by injecting contrast medium with an opacifying effect on fluoroscopy (e.g. aortography, pulmonary angiography, cerebral angiography)[7,8]. These methods offered crucial diagnostic clues to a variety of vascular and hemodynamic abnormalities, and a promise for a wide variety of diagnostic examinations as the technique for selective catheterization of visceral vessels was refined. When combining angiography with radio-opaque catheters and devices, X-ray fluoroscopy could be used as a basic means of endovascular navigation[9]. The Seldinger technique for safe vascular access further simplified the use of multiple catheters and devices in a single procedure with low risk of loss of vascular control and bleeding. Milestones in this process were the first selective coronary angiography in the 1960s[10] and the development of subtraction angiography[11,12], which increased vessel contrast and therefore the diagnostic capability of, for example, cerebral angiography[13].

The potential for direct treatment of intravascular pathology such as thrombosis and stenosis with endovascular techniques was quickly apparent. Pioneering work in this field led to the development of the intravascular balloon to widen stenosis[14,15], the stent to mechanically keep vessels patent[16,17], and the intravascular coil to embolize (block) damaged vessels[18] or aneurysms[19], among other techniques. As technical development has made catheters, stents and coils ever smaller in diameter, treatment can now be performed in some of the smallest arteries, with an inner diameter of around 1 mm[20]. One of the major strengths of the endovascular technique is the possibility to, at least in theory, quickly gain access to any artery feeding any organ while only needing to puncture a vessel at the chosen access site, such as the groin or wrist[21–24].

There is a pervasive trend across the healthcare field of adopting minimally invasive approaches to treatment and diagnostics, and the use of endovascular techniques is in line with this trend. Its main general advantages are the small incision in the body which minimizes the risk of infection, the relative painlessness which obviates the need for general anesthesia in most procedures[25,26], and the fast recovery which necessitates only a short period of post-procedure observation[27]. The endovascular approach may also in some cases be the least technically challenging, particularly for interventions in the heart and brain, which are more difficult to safely approach surgically. In comparison to other minimally invasive approaches, especially direct percutaneous puncture, the endovascular technique is more time consuming, more costly, and sometimes more technically challenging. It typically requires the use of iodine-based contrast medium, which has contraindications and some inherent risks[28]. If large devices are used, larger vascular sheaths are needed and there may be a need to use vascular closure devices and longer patient immobilization to avoid access site

complications[29]. However, the endovascular technique does have unique advantages. One is the ability to reach deep perivascular structures and lesions that may present no direct path from the skin. When performing percutaneous biopsy or lesion access for example during ablation, it is important to avoid vascular structures and traversing other organs that are not the target[30,31]. This is especially problematic for deep central liver lesions, the pancreas, the retroperitoneum and the mediastinum. All of these areas are close to large vessels and are therefore often easier to reach via the endovascular route. Another important advantage is the ability to directly diagnose and treat vascular complications of the intervention, such as dissection and hemorrhage, using angiography, embolization, and angioplasty.

2.2 TRANS-VESSEL WALL TECHNIQUE

The Seldinger technique was developed in 1953 by Sven-Ivar Seldinger[32]. The technique allows safe access to the lumen of a vessel, without significant bleeding, so that catheters and devices can be introduced and navigated to any vessel of interest for diagnostics or treatment. It involves puncturing a vessel with a needle, placing a guidewire through the needle and then exchanging the needle for a blunt vascular sheath (sometimes called an “introducer”). The introducer has a hemostatic valve that prevents bleeding from the vessel via the introducer lumen, while allowing insertion of catheters and devices such as longer guidewires for navigation, or treatment devices such as stents or coils. As such, it can be thought of as a working channel from outside the patient to the lumen of most vessels.

Inspired by this concept, our research group has developed a method that accomplishes the reverse, that is, a working channel between a luminal device and the extravascular tissue. Called the trans-vessel wall technique[33], or “extroducer”, this device is essentially an ultra-thin catheter with a sharpened end that can be sheathed. The catheter can be advanced via conventional endovascular catheters to reach the desired location of vessel exit, without damaging the catheter or blood vessel. The sharpened tip is then unsheathed, and the device is angled towards the vessel wall and advanced to penetrate into the tissue surrounding the vessel. The proximal end of the device has a connection adapter for a syringe or injection pump allowing injection of most types of solutions, such as therapeutic substances or cell suspensions.

When the injection is complete, the trans-vessel wall device can be retracted. The device tip can be detached and left in place to seal the hole in the vessel wall before the device is retracted, to prevent hemorrhage. A radio-opaque marker stays attached to the detached device tip and is used to verify that the detachment was successful, and that the device tip has not been dislodged from the vessel wall. It can also be used to find the injection site in a follow-up procedure using, for example, CT or ultrasound. In fact, if the trans-vessel wall device is used to penetrate into a lesion of interest, such as a tumor, the detached tip can be used to guide follow-up procedures, such as radiotherapy, targeting the device tip which is known to be inside the tumor.

One important application for the trans-vessel wall device is cell transplantation directly to the organ or lesion of interest. Cell transplantation is a fast-developing field in regenerative medicine and oncology, and there is increasing evidence that various types of stem cells or other cells, sometimes with bioengineered modifications, will be able to treat and even cure a variety of diseases[34]. Examples include chimeric antigen receptor (CAR) T-cells for the treatment of blood malignancy[35], CAR T-cells and stem cells for solid tumors[36–38], embryonic stem cells for macular degeneration[39], and mesenchymal stem cells for myocardial generation in heart failure[40]. Cells for transplantation are expensive to produce compared to conventional pharmaceutics and rely on advanced techniques in cell culture, gene modification and good manufacturing practices to ensure safety. Cell transplantation is also often only effective when the transplanted cells can interact directly with the organ or lesion to be treated, and may cause unwanted side effects if cells engraft in other organs or in the vasculature[41]. It is therefore imperative for both cost and efficacy that transplanted cells reach their target in high concentrations, while avoiding high rates of engraftment at other sites.

Intravenous delivery is usually the simplest route, as it only requires simple venipuncture and infusion of cells. However, this method requires either that the disease is in the bloodstream, or that the cells are able to effectively transmigrate to the target, for example, via diapedesis[42]. Even when cells are able to transmigrate, they often do so relatively non-selectively, and many cells end up in the lungs or liver[43]. A variety of other methods of delivery have therefore been employed, such as direct injection (e.g. vitreous injection in macular degeneration[39]), selective intra-vessel infusion upstream of the target (e.g. transcoronary delivery to the heart, portal vein infusion of islet cells[44]), or surgical implantation[45]. Catheter-based vascular techniques in which a relatively large catheter with a retractable needle is used to inject into the heart have been developed. However, these devices are limited to navigation in the cardiac ventricles[46] or larger vessels such as the coronary sinus and proximal cardiac veins[47]. The trans-vessel wall technique is significantly lower profile and can provide superselective direct tissue access in the distal parts of the organ vessels, where other catheter techniques may not reach, and surgical implantation is the only other option. The trans-vessel wall device has thus been studied mainly for cell transplantation[48–50]. It has been used to transplant islet cells to the pancreas in swine, and the feasibility of injections to the brain and pancreas has also been shown. We have published a more extensive review on the device discussing this, which is not part of this thesis[51]. Biopsy would be another natural application for the trans-vessel wall technique. Since the small diameter of the catheter makes it possible to reach the tissue of all organs, especially hard-to-reach areas such as the brain, pancreas, and mediastinum, it may be an attractive alternative to percutaneous techniques for biopsy of lesions within these organs. The challenge lies in designing a biopsy device small enough to be usable within the trans-vessel wall device, which has an inner diameter of 0.15 mm.

The trans-vessel wall technique is still nascent and not yet established in clinical practice. There remains room for improvements to the device design that may ease clinical integration, such as avoiding the need for tip detachment to prevent hemorrhage. Since tip detachment

renders an individual device unusable after the first injection, the cost of trans-vessel wall technique-based treatments is increased if the tip must always be detached, since a new device is needed for each injection site. There is also a need to improve navigability in distal vessels to be able to more specifically target any lesion. A lesion not reachable directly by a feeding vessel may need to be approached at an angle near perpendicular to the vessel wall. Without a way to steer the trans-vessel wall device, it is difficult to accurately penetrate the vessel wall at an angle unless the anatomy is naturally suitable. This is especially important in organs with circumferentially orientated vessels, such as the coronary arteries and veins of the heart.

2.3 THE CONCEPT OF BIOPSY

General pathology is the study of disease mechanisms, and is based on the microscopic or molecular analysis of tissues and cells from diseased organs of the body[52]. Most diseases with a known etiology and mechanism can be diagnosed by characteristic findings when performing such analyses, such as histology to detect pathological morphology or cell invasion, or molecular analysis such as polymerase chain reaction to detect disease-associated gene transcripts[53,54]. Human pathology was initially focused on the study of post-mortem subjects to define and detect these disease processes. The desire to achieve a diagnosis while a patient is still alive and can be treated led to the development of diagnostic techniques that can be placed in two broad categories: non-invasive (such as blood tests, imaging, and some physiological measurements), and invasive (such as catheter-based physiological measurements and biopsy). While non-invasive diagnostic techniques are constantly gaining ground and are preferred by clinicians and patients due to their low associated risks and typically quick results, they are rarely the reference standard for diagnosis, and rely on surrogate measurements and indirect effects of pathology[55–57].

Biopsy enables the pathologist to diagnose the disease by direct examination of the tissue and is therefore often the reference standard for pre-mortem diagnosis. It can be further categorized as excisional biopsy (i.e. surgery to remove a tumor), and incisional biopsy (i.e. selective sampling of a lesion or organ of interest using less invasive techniques). Incisional biopsy requires clear identification of the lesion by the clinician performing the biopsy. This is sometimes straightforward to perform, such as for cutaneous lesions or when the lesion is palpable beneath the skin (e.g. subcutaneous lipomas or some tumors in the breast[58,59]). When more deep-seated organs and lesions are biopsied, imaging guidance is invariably used. While they are often referred to as “minimally invasive”, image-guided incisional biopsy methods exist on a spectrum of invasiveness and complication risk, ranging from brain biopsy requiring drilling into the skull[60] to fine needle puncture of subdermal lesions, performed with local anesthetic and with a very low risk of complication. A majority of lesions in the body can be reached with direct percutaneous puncture techniques[61,62], using devices with a variety of needle diameters including large core vacuum biopsy needles (2.9 mm), core needles (1.6 mm) and fine needle aspiration (0.7 mm).

When lesions are not readily identifiable by surface examination of the patient, direct puncture is often performed by radiologists using a variety of imaging guidance techniques[63,64]. Guidance by ultrasound and computed tomography are the most common, and generally have a good success rate, depending on location and size of the lesion[30]. Digital display fusion of different imaging methods to improve targeting is gaining more widespread use[65]. However, every target organ or lesions carries specific challenges, including pneumothorax from lung biopsy[66], retroperitoneal bleeding from renal biopsy[67], traversal of sensitive organs and intestine in abdominal visceral biopsy[30], and the risk of tumor cell seeding in cancer biopsy[68]. Direct puncture may not be able to reach the organ or might be associated with increased risks. This limits the usefulness of direct puncture incisional biopsy. To improve the accuracy and flexibility of incisional biopsy, a number of different navigational techniques are employed to more easily reach the lesion of interest when direct percutaneous technique may not be the easiest and safest choice. Examples include endobronchial biopsy using a flexible bronchoscope and special devices[69], endoscopic biopsy of the gastrointestinal tract, liver, and pancreas[70,71], and endovascular biopsy techniques.

2.4 ENDOTHELIAL CELL BIOPSY

A natural target for endovascular biopsy techniques are endothelial cells and the vessel wall. Endothelial cells are a crucial component of the vasculature, lining the lumen wall of all blood vessels and acting as important barriers, mediators of endocrine signaling and hemostasis, transporters of metabolites, and regulators of vascular tone[72]. They hold importance in a wide variety of disease processes [73]. There is also increasing evidence that endothelial cells can be divided into distinct subtypes with varied phenotypes and roles in disease, and which are distributed in specific patterns in different vessel sizes and organs[74,75]. Due to these factors, there is great interest in harvesting endothelial cells from humans with different diseases and from different vascular beds, in order to further research in human endothelial cell pathology[76].

A vessel wall biopsy device was described in 1998[77]. It is a 2.33 mm diameter catheter that features a side-mounted slit and actuating cutting blade which enables cutting off a portion of the vessel wall and storing it inside the catheter. The catheter attaches to the vessel wall by suction using negative pressure from an external pump attached to the catheter. It has mainly been used to study experimental pulmonary arterial hypertension[78–80], and has not been used in humans. However, in 1999, Feng et al. demonstrated a simpler approach[81]. They found that some endothelial cells lining the vessel walls are detached and adhere to the devices used during endovascular procedures, probably due to the devices rubbing against the vessel wall. Using cell dissociation assays, the cells can then be detached from the devices to form cell suspensions that can be studied using cytology or gene expression assays. The technique has been used to study endothelial cells in patients with diabetes[82,83] and coronary artery disease[84], and to harvest cells from intracranial aneurysms[85,86]. It could also be used in drug development to study the drug concentrations in endothelial cells in both normal and pathological conditions. Endothelial cell biopsy is not yet used in clinical practice, but as

knowledge of endothelial cell subtype variation and pathology develops, it may become a useful clinical tool to aid prognosis or guide treatment decisions[86]. This may be especially true in intracranial vascular disease, where the increasing use of interventional devices to treat cerebrovascular conditions makes brain endothelial cells from patients with complex disease panoramas available for biopsy. This includes conditions such as intracranial aneurysms treated with coiling, and ischemic stroke with treated mechanical thrombectomy. The advent of mechanical thrombectomy to treat acute ischemic stroke has led to the availability of thrombi gathered from the brain vasculature, making thrombus analysis in stroke a developing research field[87–90]. Thrombus analysis is particularly interesting in the investigation of cryptogenic stroke, that is, when the embolic source is unknown. However, the endothelial cells lining the vessel from which the thrombus was removed are also likely to contain important pathological changes that could be detected using the modern molecular analysis methods, described in section 2.7.2.

2.5 ENDOMYOCARDIAL BIOPSY

The first type of endovascular biopsy to be developed was endomyocardial biopsy (EMB), which is still the most commonly used technique today. Developed in Japan in the 1960s[91], the technique is based on a forceps that sits at the end of a semi-flexible braided wire, which with a handle that allows it to be opened and closed. It is introduced into a catheter which has been placed in the right ventricle via the internal jugular vein. Endomyocardial biopsy can be used to diagnose disease affecting the myocardium, such as myocarditis, infiltrative cardiomyopathy, intracardiac masses, and transplant rejection after heart transplantation[92]. Transplant rejection is the primary indication for endomyocardial biopsy, especially in the first year after transplantation when the risk of rejection is the highest[93], and so EMB is performed routinely up to 15 times in the first year and less frequently thereafter[94]. The symptoms of rejection can be very mild and non-specific, and early diagnosis is important to effectively guide immunosuppressive treatment[95]. Non-invasive methods of detecting transplant rejection have been developed[96–99], but EMB is still considered necessary to confirm the diagnosis[100,101].

The complication risk of endomyocardial biopsy is variably reported as between 1% – 8.9%[92,102,103]. The most common complication is arrhythmia, as manipulation with the device in the ventricle, particularly when rubbing against the tricuspid valve or stretching the ventricle wall when taking the biopsy, stimulates the conduction system of the heart[104]. Most arrhythmias are transient and self-terminating or can be effectively treated with medication during the procedure[105]. However, persistent ventricular arrhythmia and death have been reported. Other important complications include perforation of the ventricular wall causing tamponade, which can also be fatal; damage to the chordae tendineae causing valve dysfunction[106,107]; and thrombus formation on the endocardial surface damaged by the forceps. Thrombus formed in the ventricle can embolize to the lungs (in right ventricular biopsy) or to the brain causing stroke (in left ventricular biopsy or right ventricular biopsy in patients with septal defects)[108]. Repeated biopsies, as performed in heart transplant

monitoring, require repeated traversals of the tricuspid valve and are associated with tricuspid regurgitation, which can cause serious morbidity[109].

The standard technique, and also overwhelmingly the most used, is right ventricle biopsy via transjugular access[110]. This path to the heart is relatively straight, though uncomfortable for the patient due to the vascular access site on the neck; moreover, it is associated with a larger radiation dose for the interventional cardiologist compared to other access sites. It is the least technically complicated and places the least demands on flexibility and steerability of the device, as the right jugular vein presents a largely straight path to the right ventricular septum. Since it is a transvenous technique, there is less consequence of perforation and embolization, as the low pressure means tamponade builds slowly and can be treated, while embolization to the lungs is more often clinically silent and generally easier to treat than ischemic stroke.

Biopsies are preferably taken from the interventricular septum[111], as it is much thicker than the rest of the right ventricular wall, which is prone to perforation. This means that sampling various parts of the heart is difficult and generally avoided, even though there is good reason to try to achieve varied sampling from a diagnostic perspective[112]. The first reason for this is that the disease process one wishes to discover may not always neatly occur in the right ventricular septum, but may rather have a patchy distribution, as occurs in storage diseases such as amyloidosis and sarcoidosis as well as infectious diseases such as viral cardiomyopathy[108,112]. Disease may also preferentially occur in the left ventricle, which is completely missed with right ventricular endomyocardial biopsy.

To address this problem, left ventricular endomyocardial biopsy has been explored[108,112,113]. While it has been deemed safe in some studies[112], it is generally avoided due to the specific complication risks outlined above, which are thought to outweigh the benefits. Left ventricular biopsy is typically performed by femoral artery access, since carotid artery access is associated with increased risk of stroke. Femoral artery access requires traversing the aortic arch, which requires a more flexible bioptome and may hinder steerability in the left ventricle. Recently, there have been case series reporting radial artery access for left ventricular EMB[114–116]. Radial access is associated with fewer access site complications and shorter in-hospital stay, though it has mostly been studied for coronary angiography and percutaneous coronary intervention[117], which uses different devices than endomyocardial biopsy. Nevertheless, moving to radial or antecubital vein access as routine could improve the safety profile of EMB.

2.6 OTHER METHODS OF ENDOVASCULAR BIOPSY

Some devices and indications for other endovascular biopsy methods are in clinical use. Endomyocardial biopsy forceps have been used to biopsy intravascular lesions[118], most commonly pulmonary artery sarcoma, as the proximal pulmonary arterial trunk can be readily reached from the right ventricle. Pulmonary artery sarcoma is a rare malignant neoplasm that originates from mesenchymal stem cells in the pulmonary artery and largely grows into the

vascular lumen, making it suitable for endovascular biopsy[119]. Since the pulmonary artery is readily catheterized for pressure measurements, the same technique can be used with a small endomyocardial biopsy forceps to sample most proximal pulmonary artery contrast filling defects detected on CT or angiography[120]. Technical problems may arise when the lesion is more distal in the pulmonary vasculature, or if it does not grow sufficiently into the lumen to partially occlude it. If the growth is along the vessel wall, it may be difficult to direct an endomyocardial bioptome to the lesion to get a good grasp of it[121]. There are reports of using EMB devices to biopsy other cancerous lesions invading the vessels, such as renal cell carcinoma[118].

The liver is another common target for biopsy, either due to growth of suspicious intrahepatic lesions, or to sample normal parenchyma to diagnose or assess damage in primary liver disease or in transplanted livers to diagnose autoimmune rejection[122]. Direct percutaneous biopsy (usually but not always with ultrasound guidance) is the most common method due to its simplicity[123], as the right lobe is easily accessible from the skin. When the lesion is not accessible from the skin, percutaneous biopsy fails, or the patient suffers from coagulopathy, transjugular biopsy is usually performed[124,125]. The device used is similar to the percutaneous needle, but longer and somewhat flexible. The path to the liver from the jugular vein is relatively straight, so navigation is not difficult. Liver parenchyma may also be sampled by gastroscopy using a fine needle aspiration technique[71]. The liver can be reached via the stomach or duodenal bulb, and ultrasound is used for guidance. The location of the lesion and patient characteristics, including their need for additional endovascular or endoscopic procedures, will dictate the most suitable route for liver biopsy.

Like the liver, the kidneys are often biopsied, usually via the direct percutaneous route[126]. However, in patients with contraindications to percutaneous biopsy, the transjugular technique is also available[124]. Transjugular liver and kidney biopsy are considered safer, specifically with respect to bleeding, as blood will mostly return to the venous system. It can be combined with arterial coiling of the artery feeding the biopsied area in the same session to reduce the risk of bleeding, which is an advantage of endovascular biopsy techniques over direct percutaneous techniques.

2.7 BIOPSY ANALYSIS

2.7.1 Histopathology and immunohistochemistry

As discussed above, histopathology defines most diseases via the characteristic morphological patterns revealed by tissue staining and microscopic evaluation. Tissue and cells from biopsy are therefore typically analyzed by histopathological section and staining in order to determine a diagnosis or grade the severity of disease. Different stains color different structures of the cell walls, nuclei, cytoplasm, and interstitium. Each stain has different uses depending on the diagnostic question such as hematoxylin-eosin as a general purpose stain and Prussian blue for hemosiderin deposits in iron storage diseases[127].

Additional tissue section imaging techniques are used when standard stains do not produce clear differentiation between healthy and pathological tissue, or when certain phenotypes need to be quantified. Examples include immunohistochemistry, which creates a color stain reaction on the tissue section where proteins of interest are expressed, with color intensity typically reflecting the amount of protein expressed[128]. This can be used to qualitatively examine which cell types are present in the tissue by means of characteristic protein marker expression, and to semi-quantitatively examine expression of proteins related to aspects such as cell proliferation, hormone receptor expression, and mutations relevant to cancer pathology[129]. Tissue sections may also be examined using other techniques, such as transmission electron microscopy[130] and in situ hybridization techniques[131]. Transmission electron microscopy is typically used to evaluate the presence of abnormal intracellular deposits in infiltrative diseases such as amyloidosis[132]. Some examples of important diagnostic outputs from histopathology include determining the presence of cancer and the subtype of the cancer, and grading the level of acute rejection of transplanted hearts. Subtyping of cancer has prognostic implications and affect treatment decisions, such as distinguishing between ductal and lobular carcinoma in breast cancer. The International Society for Heart and Lung Transplantation specifies three grades of rejection depending on the extent of leukocyte infiltration, myocyte damage, and the presence of edema, hemorrhage, or vasculitis[133].

Since the analysis of biopsies using histopathology requires fixation, often paraffin wax embedding, and sectioning onto multiple slides to perform the various techniques described above, tissue biopsies need to be large enough to properly section several times and may not be excessively deformed by the biopsy procedure[134]. Endomyocardial biopsy, for example, frequently causes crush artifacts due to the bioptomes jaw-like forceps, which makes part of the tissue unsuitable for analysis as the morphology of the tissue is distorted[110]. Analysis of very small amounts of cells, for example as gathered from fine needle aspiration, is called cytopathology, and similar techniques apply as in histopathology. Diagnosis via this route is much more difficult, as cells are not examined *in situ* in the tissue, but rather dissociated and placed on a glass slide in a random configuration. Cells may also be kept in suspension and then examined using flow cytometry and fluorescence-activated cell sorting (FACS)[135].

Despite tailored analysis methods for very small cell samples, the diagnostic yield of cytological methods is generally thought to be lower than that of histopathology. To increase diagnostic yield of both tissue and cell samples, there is an increasing interest in the development and clinical use of molecular pathology methods, which use molecular biology assays to investigate tissues with regards to DNA, gene expression, and protein content. Polymerase chain reaction has been used to detect the presence of, for example, viral genome in a sample to indicate infection[132,136]. Still, molecular methods have long been limited to investigation of one or a few molecular markers of disease, which has precluded gaining comprehensive diagnostic information from a small sample using molecular assays rather than morphology-based methods of histology and cytology. This paradigm may be changing

however, with the advent of high-throughput assays of molecular markers, called “omics” methods.

2.7.2 Omics technologies and molecular pathology

“Omics” is a word initially derived from genomics, as in study of the complete genome of organisms. It refers to the study of comprehensive or even complete surveys of biological molecules in cells or tissue samples[137]. Recent developments in molecular biology, particularly in nucleic acid sequencing, have dramatically reduced the costs of detecting and quantifying biomolecules[138,139], while increasing potential coverage in a given sample. “Next generation sequencing” refers to these methods, such as sequencing-by-synthesis, where all DNA (the genome) or all transcribed RNA (the transcriptome) can be sequenced and quantified to a high degree of certainty[140,141]. In the case of the transcriptome, quantification is of paramount importance to yield useful information on the biological state of a sample. Quantification was initially relative, but more recent techniques offer semi-direct quantification of the number of each RNA transcript present in a sample[142,143].

Omics as an umbrella term also includes other biomolecule sets, such as proteomics, which deals with the translated protein content in a sample; and metabolomics, which concerns metabolites like chemical substrates. The development of omics technologies has been explosive in the 21st century, and new techniques with greater sensitivity and increased robustness are constantly being developed. A major development in the last 15 years was the decrease in sample size needed to perform robust RNA sequencing (RNA-seq), resulting in true single cell sequencing in 2009[144]. The possibility of omics technologies to improve diagnostic testing was quickly realized by the oncology and clinical genetics communities[145]. This was a natural development, as DNA sequencing (DNA-seq) was the first technique to mature, and tumor heterogeneity with regards to DNA mutations is a well-known problem when trying to make clinical predictions in oncology[146]. Moreover, the genome is the main interest of the discipline of clinical genetics, and DNA-seq may detect novel inherited diseases and deepen our understanding of diseases with a known genetic component[147].

The microarray can be considered the first transcriptomic technique to be developed. In this method, high-throughput multiplex PCR reactions are performed to survey a large number of gene transcripts in a single sample[148]. This led to a great interest in using transcriptomic signatures or “gene expression profiles” of diseased tissues and cells to diagnose disease and offer clinical prognosis, since most disease processes can be characterized by modified expression of transcripts in the tissue affected[149,150]. There are several possible causes for this modified expression pattern, including a reaction of the constituent cells of the tissue, such as in macrophage activation[151]; infiltration of cells such as lymphocytes into the tissue[152]; or transcripts generated by pathogens, such as the transcription of viral RNA in an infected cell[153]. A large number of general and specialized microarray kits are commercially produced, which can be used to survey the expression of thousands of genes in a sample[154].

RNA sequencing allows for less biased discovery and quantification of these kinds of transcriptional signatures, since prior knowledge of the exact transcripts and transcript isoforms that one wishes to detect is not needed. Instead, the RNA-seq assay can be designed to detect and quantify transcripts with a certain minimum concentration (possibly down to a single transcript), or a certain family of RNA (e.g. mRNA, long non-coding RNA, and microRNA) in a comparatively unbiased manner[155]. As RNA-seq has become more robust and sensitive, interest in RNA-seq based diagnostic tests has increased among clinical researchers, as gene expression changes in disease are more likely relevant to clinical medicine, in comparison to the genomic landscape studied in DNA-seq.

RNA sequencing encompasses a variety of techniques, but the most commonly used is short-read sequencing-by-synthesis on the Illumina platform[155]. Briefly, total RNA is isolated from a cell or tissue sample, and then fragmented. cDNA is synthesized from RNA fragments using reverse transcriptase, and adaptors are added to the cDNA. Non-ribosomal RNA is enriched by PCR amplification to form a sequencing library with multiple single-stranded cDNA copies of each transcript. The library is then sequenced by reading the strands using the Illumina sequencing-by-synthesis technology: the single-stranded DNA is attached to a flow cell, and DNA polymerase synthesizes a complementary DNA strand using fluorescent bases, which are detected optically. The read length (ca 50-500 base pairs), and the number of reads across samples ("sequencing depth") are pre-specified. Samples are typically sequenced to 5–30 million reads each, with detection rate of rare transcripts increasing with sequencing depth at an increased cost per sample. The data are processed in multiple steps, and incomplete and low-quality transcripts are excluded. The remaining transcripts are mapped to a reference genome. Longer read length and paired-end reading will reduce the number of transcripts that cannot be unambiguously mapped to the genome. The number of transcripts mapped to each gene will provide quantification. If longer reads are employed, the chance of detecting transcriptional isoforms improves[143,155].

After mapping, normalization and filtering is performed on the data. Differential gene expression analysis is then typically performed to highlight transcriptomic differences between samples[155]. The RNA-seq experiment can be designed in a large variety of ways in order to examine different aspects of the transcriptome, from bulk quantification of expressed mRNA to detection of rare isoforms and RNA varieties. The RNA sequencing pipeline has a number of sources of error and technical bias, which influence detection and true quantification of transcripts and must be considered during analysis. Examples include bias toward amplification of longer mRNA, increased mappability of highly unique genomic regions, and under-detection of transcripts with atypically high or low content of the nucleotides G and C ("GC-bias")[156].

While omics techniques do bring promises of more biological data from small samples than previously possible, the techniques have some drawbacks, which may have slowed down widespread development and adoption of omics-based diagnostic tests in clinical practice[157]. The wealth of biological data produced by omics techniques meant that new analysis methods

and statistical methods needed to be developed in the field of bioinformatics, and this development is still ongoing[158,159]. There may be special requirements for the clinical handling of biological samples destined for omics analyses, and there are ethical and privacy concerns with such all-encompassing biological analyses of patients' tissues and cells[160,161]. The growing field of translational omics and "omics-based clinical trials"[162,163] seeks to address these issues through assay design, study design, validation procedures, and specific ethical and regulatory practices. Another reason for slow adoption of omics in the clinic is the fast-moving technology and specific equipment required for sample preparation, sequencing, and data handling, which is currently not widely available at hospitals[164]. Nevertheless, omics technologies are likely the future of medical molecular pathology and will play a role in the diagnosis and prognosis of disease, supported by the continued use of tissue and cell biopsy.

2.8 PERSONALIZED MEDICINE AND MINIMALLY INVASIVE INTERVENTIONAL TECHNIQUE

Personalized medicine, or precision medicine, is a term given to the notion of increased specificity in medical treatments, where the treatment a patient is given is tailored as closely as possible to the unique genetic and phenotypic makeup of the patient and their disease[165,166]. Omics technologies have deepened the understanding of pathophysiological processes with implications for treatment, especially in cancer[167,168], and several new treatments and optimizations of current treatments based on genetic and transcriptional profiles are currently in the clinical trial phase or even approved for clinical use. Omics have been used to design tests that predict response to drugs based on the patient's genotype (pharmacogenomics)[169]. Prediction of the response to cancer treatment has also been explored based on the gene expression profile of a tumor; one example of this is the MammaPrint test, which uses a 70-gene signature for breast cancer[170]. Tumor cells can also be found circulating in the blood stream, and these could be sampled using simple blood draw and analyzed with omics techniques to monitor tumor behavior over time.

Molecular tests will thus increasingly be used to select the most appropriate treatment and used throughout a treatment plan to monitor treatment effects and thus enable personalized adjustments of treatment plans. Initially, whole genome (or exome) sequencing of the patient will provide a baseline genetic profile, and a biopsy of the organ or lesion will be necessary to provide initial transcriptomic, metabolomic and gene mutation profiles on which to base treatment decisions. Both initial diagnosis and serial monitoring in personalized medicine will therefore often require biopsy. The omics profile of the patient and disease will not only inform treatment selection but can be used to customize the treatment to the patient, for example using CAR T-cells[35,171,172] or tailored gene therapies[173]. Delivering these therapeutic substrates to the target lesion or organ at a sufficient concentration remains a challenge[174,175], as many cell and gene therapies are ineffective when delivered intravenously. Direct delivery to the target organ using imaging-guidance, either by needle or by catheter injection is therefore increasingly used in clinical trials[175,176].

Thus, both highly targeted biopsy and treatments will be important cornerstones in personalized medicine[177–179]. Interventional radiologists and cardiologists will rely on the continuous development of effective minimally invasive methods to perform biopsy and treatments. Interventions based on endovascular techniques may facilitate some of these methods.

3 RESEARCH AIMS

The overall aim of this thesis was to develop novel devices and techniques of superselective tissue access and biopsy using the endovascular route, and to employ modern molecular methods, specifically RNA sequencing, to analyze the biopsy samples generated by these techniques.

We specifically aimed to investigate:

- Whether endothelial cells could be harvested during mechanical thrombectomy using a stent retriever device, and whether the cells could be expanded by cell culture and analyzed using single cell RNA sequencing (Study I).
- Whether the trans-vessel wall technique for endovascular tissue access could be improved to reduce the risk of hemorrhage and other complications, and to allows access to sites in the body that are difficult to selectively and safely reach, such as the subepicardium, kidney capsule, and pancreas (Study II).
- Whether a novel endomyocardial biopsy device could be developed that was smaller and more flexible, and therefore less traumatic and more steerable, to improve safety and precision in endomyocardial biopsy. We also wanted to investigate the use RNA sequencing for analysis of the tissue samples from the device (Study III).
- Whether the device developed in Study III is safe to use in an animal model of acute myocardial infarction, and whether RNA sequencing analysis of biopsy samples could detect gene expression changes caused by the infarction (Study IV).

4 MATERIALS AND METHODS

4.1 STUDY DESIGN

This thesis explores three main technologies: endothelial cell harvest in mechanical thrombectomy (Study I), the trans-vessel wall technique (Study II), and micro-endomyocardial biopsy (Studies III and IV).

4.1.1 Study I

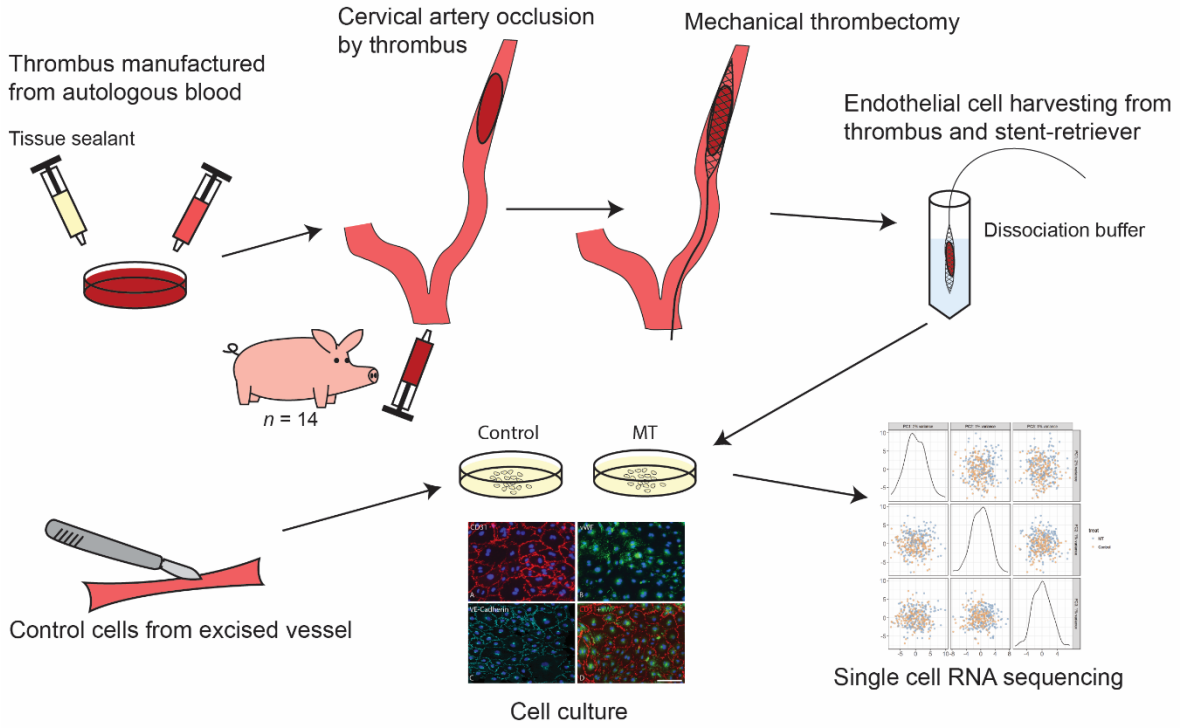

Figure 1: Design of Study I. MT = mechanical thrombectomy.

An experimental study was performed in 14 swine. Thromboembolism was induced in the cervical arteries and mechanical thrombectomy (MT) using a stent retriever was performed. After each thrombectomy pass, the thrombus was removed, and endothelial cells were harvested from the devices and thrombus. The cells were enriched, cultured, and analyzed using immunofluorescence and single cell RNA sequencing. We evaluated the robustness of sequencing data, and whether any difference could be detected between control cells and MT. Finally, we analyzed the stent retriever used in the procedures using scanning electron microscopy (SEM) to look for any potential damage caused by the harvesting procedure.

4.1.2 Study II

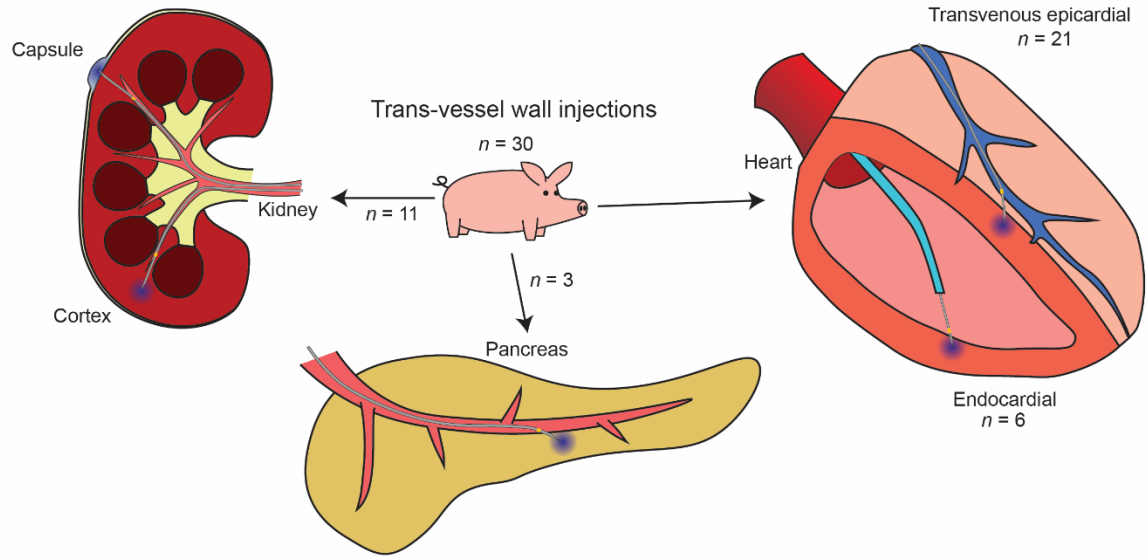

Figure 2: Design of Study II.

We performed a swine study of trans-vessel wall injections of methylene blue in the heart (epicardially and endocardially), kidney (capsule and cortex), and pancreas. Injections were initially performed in non-follow up interventions. Follow-up was then performed for epicardial heart (1-3 days) and kidney (14 days) injections for further evaluation of safety. We evaluated the safety during procedures, during follow-up, and using necropsy. Necropsy was also used to evaluate the efficacy of the injections in heart and kidney to verify that the correct area had been reached, and that the spread of injected material was sufficient.

4.1.3 Study III

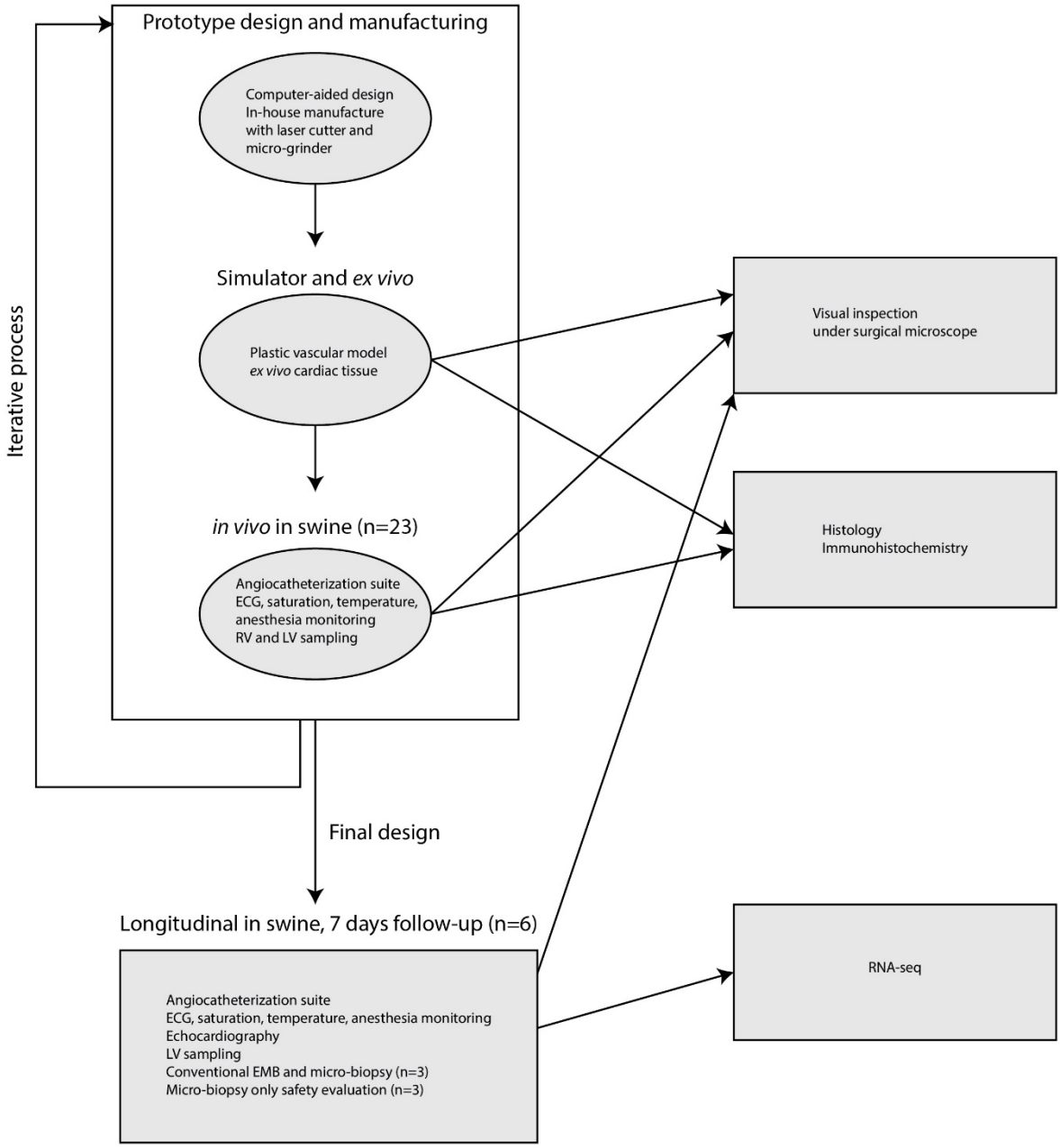

Figure 3: Design of Study III. ECG = electrocardiography, RV = right ventricle, LV = left ventricle, RNA-seq = RNA sequencing.

We developed a novel endomyocardial biopsy device (micro-EMB) and evaluated it in simulator, *ex vivo*, and *in vivo* in swine in both non-follow-up and longitudinal studies. Swine studies were performed to evaluate the feasibility and safety of using the device *in vivo*. We analyzed biopsy samples using histology, immunofluorescence, and RNA sequencing. RNA sequencing experiments were performed to evaluate the technical robustness and specificity of mRNA transcriptomes generated from the micro-EMB samples.

4.1.4 Study IV

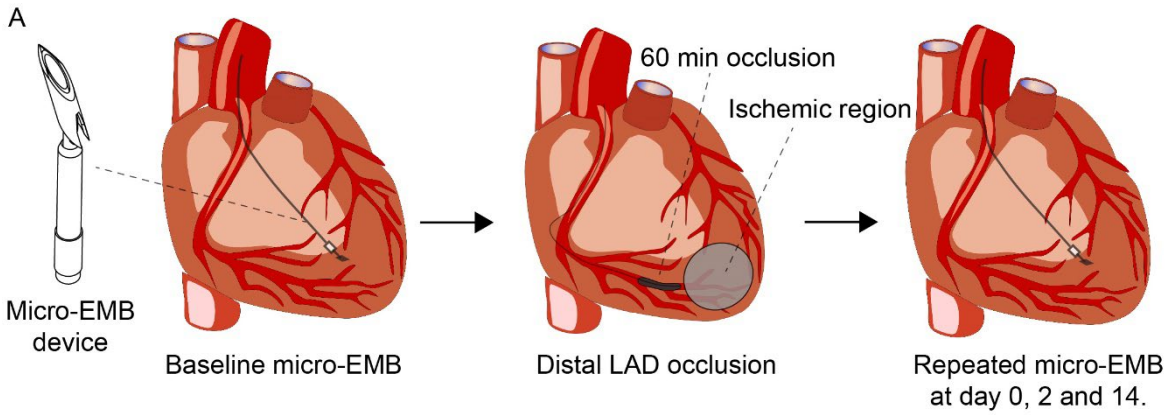

Figure 4: Design of Study IV. micro-EMB = micro-endomyocardial biopsy, LAD = left anterior descending artery.

We used the micro-EMB device in a swine model of acute myocardial infarction (AMI) with 14 days of follow-up. Myocardial infarction was induced using endovascular balloon occlusion for 60 minutes followed by reperfusion. Micro-EMB samples were taken before occlusion, during occlusion, after occlusion for up to 3 hours, and at days 2 and 14. Samples were taken from the area of myocardial infarction and from an unaffected area of the left ventricle. (LV) We evaluated the safety of using the micro-EMB device in a diseased heart by performing RNA sequencing on samples from all time-points and evaluating the data for transcriptional changes caused by the infarction.

4.2 ETHICAL CONSIDERATIONS

All studies were conducted in accordance with both national guidelines and the local guidelines at Karolinska Institutet. Studies were also approved by the regional ethics board for experimental animal studies, Norra Stockholms Djurförsöketiska nämnd (approval documents: N140/14, N7/15 with amendment N49/16, and 525-2018). All animal research was conducted according to the ARRIVE guidelines[180]. Across all four studies, a total of 76 swine were used, 20 of which were part of longitudinal follow-up studies.

Research on and clinical translation of invasive medical devices and technologies inevitably involves ethical concerns regarding the utilization of experimental animals and patients as research subjects[181]. The use of experimental animals for the development and validation of medical devices is often necessary (and, as mentioned above, regulatory requirement), and particularly when it comes to FDA Class II-III devices[182] such as endovascular catheters and biopsy devices. Even if animal studies were not a regulatory requirement, it is considered ethically prudent to prioritize the safety of patients over a limited number of animals participating in pre-clinical and translational trials of new medical devices.

We strove to minimize the use of experimental animals in accordance with the 3R paradigm; that is, replace, reduce, refine. We replaced initial testing with computer and technical *ex vivo* simulation where possible, to verify the basic function and behavior of the devices. However, large animal studies were required to verify the intended functionality and safety of the devices and their intended use, since it was crucial to closely simulate the human situation

with regard to the size of blood vessels, physiological and biochemical parameters, and compatibility of the devices with existing technologies used in clinical practice, such as clinical grade catheters and X-ray interventional equipment. We minimized the number of animals used and refined the procedures as much as possible. For example, when possible, injections were performed in more than one organ per animal (Study II). We used general as well as local anesthesia whenever interventions were performed. During all experiments we had dedicated personnel for the monitoring and management of anesthesia and analgesia. Before and between interventions, animals were cared for by staff specifically trained in large animal husbandry, under veterinarian supervision.

4.3 DEVICE DEVELOPMENT AND PROTOTYPING

Studies II–IV concerned custom devices. They were designed using computer-aided design software (Solid Edge) and manufactured in-house using a specialized laser micro-machining laboratory. Extruded nitinol tubes, wires and other materials were purchased, machined, and assembled to create the different devices.

4.3.1 Trans-vessel wall device.

In Study II, we modified the previously described trans-vessel wall device[33]. Briefly, the device consists of a very thin (<0.2 mm) tapered nitinol catheter with a sharp needle-like distal end. Proximal to the tip, a radio-opaque collar sit at the distance of intended penetration depth, followed by a short detachment zone and another radio-opaque marker. The tip is detached by electrolysis. The sharp nitinol catheter is housed in a polytetrafluoroethylene catheter to avoid unintended damage to the co-axial catheter used for navigation, and unintended vascular injury when the device is in the vascular lumen. The original device was altered to improve penetration characteristics. The force required to penetrate a latex membrane was compared between the previous design and the modified design. The detachable tip mechanism and the most proximal radio-opaque marker were removed, as tip detachment to prevent clinically significant hemorrhage was hypothesized to be unnecessary in the organs targeted (heart, kidney, and pancreas).

4.3.2 Micro-endomyocardial biopsy device

We created a novel device to perform endomyocardial biopsy, the “micro-endomyocardial biopsy device” (micro-EMB), designed to feature a fundamentally different sampling mechanism than in conventional EMB, which uses a forceps-like action. The device consists of a movable cutting tip with prongs protruding in the proximal direction (Figure 5a). The shaft of the device is a nitinol tube sharpened circumferentially at the distal end. The tip is attached to a wire that passes through the tube, which makes it possible to move it forwards and backwards by hand to accomplish the sample harvesting action. The tissue is cut off by the prongs of the tip and the sharpened end of the tube and stored in a small slot in the tip. The device is designed to be housed in a micro-catheter to prevent damage to the guiding catheter.

4.3.3 *Ex vivo* simulator

For endomyocardial micro-biopsy development (Study III), a simulator was constructed from polyethylene tubing and acrylic glass. The simulator encompassed a closed system filled with saline, into which catheters and devices could be introduced. It was designed to simulate femoral access to the aorta and left ventricle of the heart, which consisted of a small acrylic glass chamber. Left ventricular tissue from swine was placed in the chamber, which allowed a simulation of the biopsy procedure with complete visual control, yielding a true tissue sample. This model was used to optimize the device design before *in vivo* testing.

4.4 ANIMAL EXPERIMENTS

4.4.1 Anesthesia

Animal experiments were performed with mixed breed Yorkshire-Swedish farm swine, due to the similarity of their vascular system to that of humans (Studies I–IV). Animals were first sedated using intramuscular injection of tiletamine 2.5 mg/kg, zolazepam 2.5 mg/kg and medetomidine 0.1 mg/kg. Anesthesia was then induced with injection of propofol or sodium pentobarbital, after which the animals were intubated and mechanically ventilated. Anesthesia was maintained with pentobarbital infusion or by inhalation of isoflurane or sevoflurane, and analgesia was managed with hourly fentanyl injections. Induction, maintenance, and recovery from anesthesia, as well as analgesia, were continuously supervised by dedicated staff experienced in large animal anesthesia and husbandry. During the procedures, we continuously monitored ECG, body temperature, oxygen saturation, urine production, and invasive blood pressure. For interventions in the heart (Studies II–IV), animals were pre-treated with 75 mg i.v. amiodarone in up to two doses depending on experiment duration. After interventions, animals were either sacrificed by an i.v. sodium pentobarbital injection (Studies I–IV), or awoken from anesthesia, extubated and returned to animal housing (Study II–IV). In Studies II–IV, necropsy was performed to evaluate the effects and complications of the new device interventions.

4.4.2 Husbandry

Before the interventions, the animals were housed for at least a week in laboratory adjacent animal housing. In the longitudinal studies (Study II–IV), the animals were monitored for complications by husbandry staff between interventional sessions. In Study IV, periprocedural analgesia was achieved with intramuscular meloxicam injections (0.4 mg/kg/day).

4.5 INTERVENTIONS

Interventions were performed in a clinical grade interventional suite (Philips Allura XD20) guided by X-ray fluoroscopy. Vascular access was obtained by placing an introducer sheath (5–8.5 French) in the femoral arteries (Studies I–IV), right femoral vein (Study II) or the right jugular vein (Study III) using either an ultrasound-guided modified Seldinger technique, or surgical cut-down technique. For the longitudinal experiments, the modified Seldinger

technique was used exclusively, and smaller sheaths (5–6 French) were employed. Endovascular navigation was achieved using standard clinical equipment including diagnostic and guiding catheters, standard and hydrophilic guidewires, micro-catheters and micro-wires (Studies I-IV as well as steerable sheaths (Studies II and III).

4.5.1 Thrombectomy endothelial cell sampling (Study I)

In Study I, we performed endovascular thrombectomy to simulate the treatment of large vessel occlusion ischemic stroke in 14 swine. Thrombi were manufactured by drawing blood from the animal, mixing it with tissue sealant (containing factor VIII, aprotinin, and fibrinogen) to induce clotting, and cutting the clotted blood into elongated thrombi using scalpel and forceps. A guiding catheter was then placed in the external carotid artery or the subclavian artery, and the thrombi were loaded into the catheter and injected into the vessels with a saline flush to induce thromboembolic occlusion. The vessels were left occluded for 0.5, 3, or 6 hours to simulate clinical stroke scenarios where time to reperfusion is variable. Mechanical thrombectomy was then performed according to clinical routine, using a Solitaire stent retriever device. In some cases, the thrombectomy procedure was carried out in patent vessels where no thrombus had been injected, to test the ability of the device to gather endothelial cells without a thrombus present. In two of the animals, positive controls were generated by mechanical removal of endothelial cells from a surgically excised femoral artery branch.

4.5.2 Trans-vessel wall technique injections (Study II)

The trans-vessel wall device was navigated to the heart (n = 21), kidney (n = 11), and pancreas (n = 5), and injections of iohexol contrast and methylene blue were performed. A small amount of contrast (< 100 µl) was injected to confirm that the trans-vessel wall device tip had reached the target site, and then 250 µl of methylene blue was injected to simulate the injection of a therapeutic substance or cells. The distribution of blue discoloration of the tissue was then examined on necropsy. A steerable micro-catheter (SwiftNINJA, Merit Medical) was used for the epicardial and pancreas injections in order to improve targeting. In the heart, injections were performed in the subepicardium (n = 93 injections) using a transvenous approach via the coronary sinus to the cardiac veins, as well as the endocardium (n = 20 injections) via femoral arterial approach to the left ventricle. Eight animals in which epicardial injections were performed were monitored for one day (n = 3) or three days (n = 5) before sacrifice. Kidney injections were performed using a transarterial approach in eleven animals, three of which were followed for 14 days. We monitored urine and blood biochemistry for markers related to kidney injury at baseline, day 2, day 7, and day 14 in these animals. Injections were targeted to the renal cortex and subcapsular space. For the pancreas, ten injections were performed in five animals with no follow-up.

4.5.3 Micro-endomyocardial biopsy (Study III)

In Study III, different iterations of the micro-EMB prototype were tested in vivo (n = 23). Initial tests were performed in the right ventricle (RV), and the most effective versions were

also tested in the left ventricle. The final design, deemed most promising with regards to effectiveness, safety, and ease-of-use was used in the same manner, without alterations, in Studies III and IV. The micro-EMB device was loaded tip to tip in a 2.7F micro-catheter (Renegade Hi-Flo, Boston Scientific, or Carnelian, Tokai Medical). We navigated to the region of interest of the left or right ventricle using a steerable sheath, diagnostic catheter or guiding catheter. The micro-catheter was then advanced co-axially until a connection to the ventricular wall was felt and seen on fluoroscopy. To perform the biopsy, the micro-EMB device was then advanced, penetrating the endocardial wall. The tip portion of the device was then advanced further and rotated within the myocardium, before being retracted. Finally, the entire device and micro-catheter was then withdrawn to collect the sample. In Study III, samples were collected from varying parts of the left and right ventricle.

4.5.4 Longitudinal study of micro-endomyocardial biopsy in healthy swine (Study III)

As part of Study III, a safety study was performed in three animals using micro-EMB only. In an additional three animals micro-EMB was used in conjunction with conventional endomyocardial biopsy (using clinically available devices from Cook Medical or Argon Medical) to gather samples for RNA sequencing. All animals underwent transthoracic echocardiography at day 0 and day 7 to evaluate left ventricular ejection fraction and any signs of complications, such as pericardial effusion.

4.5.5 Acute myocardial infarction by balloon occlusion (Study IV)

In Study IV, myocardial infarction was induced by placing a balloon catheter in the left anterior descending artery beyond the third diagonal arteries, inflating it for 60 minutes, then deflating it. Complete occlusion and reperfusion were verified by angiography. Micro-EMB was performed at baseline, during occlusion, and after reperfusion at day 0 up to 3 hours after occlusion and on days 2 and 14. Biopsies were taken from the LV ischemic apex area and from the lateral LV, away from the ischemic region, using fluoroscopic guidance to select the biopsy sites.

4.6 TISSUE ANALYSIS

4.6.1 Endothelial cell handling and analysis (Study I)

After withdrawing the stent retriever from the body, the blood clot was manually removed with tweezers. The device was then submerged in endothelial cell dissociation buffer for 5 minutes, with gentle shaking of the device and brief vortexing. The buffer, now containing the dissociated cells, was centrifuged for 5 minutes at 300 G, and red blood cell lysis buffer was then added for 5 minutes on ice, followed by another centrifugation. Cells were then washed with PBS and centrifuged again. Cells were then cultured on standard culture plates with endothelial cell growth medium (EGMV2, replaced every 3-4 days), with the addition of penicillin, streptomycin, and amphotericin B. The cells were passaged using trypsin/EDTA when near confluence. Cultured cells were fixed with 4% paraformaldehyde in PBS, blocked

with serum-free protein blocking solution, and incubated with anti-CD31, anti-vWf and anti-VE-cadherin primary antibodies. After incubation with secondary antibodies, cells were mounted with DAPI-containing mounting medium and imaged with confocal microscopy (LSM700, Carl Zeiss).

4.6.2 Single cell RNA sequencing (Study I)

Cells from thrombectomy and control EC cultures were sorted in a 384-well plate using fluorescence activated cell sorting (FACS). After incubation in a fluorescence-tagged primary antibody cocktail of CD31, CD146, and DAPI, cells were resuspended in FACS buffer sorted using a BD FACSJazz cell sorter. Single cell sequencing was then performed using the Smart-seq2 protocol[183] on the Illumina HiSeq platform, at 1x50 base pairs.

4.7 ENDOMYOCARDIAL BIOPSY SPECIMEN ANALYSIS (STUDIES III AND IV)

After the micro-EMB device was retracted from the myocardium, it was removed from the patient and inspected under a surgical microscope. The size and gross quality of the tissue was evaluated and scored on a scale from 0 to 4, where 0 represented no visible sample, and 4 represented a large sample protruding from the sample collection notch.

4.7.1 Histology and immunofluorescence (Study III)

Microscopy was used to verify that samples were of myocardial origin. Micro-EMB tissue specimens were placed on slides and stained either with hematoxylin-eosin, with May-Grünwald-Giemsa stain, or by immunofluorescence for troponin I with DAPI used as a counterstain.

4.7.2 RNA isolation and sequencing (Studies III and IV)

Samples intended for sequencing were immediately snap frozen and stored in PCR tubes at -80° C. In Study III, whole blood and skeletal muscle samples were acquired to use as controls for tissue differentiation. Samples were processed with a modified protocol based on the Agencourt RNAdvance Tissue kit. Reagent volumes were scaled by 1:13, and homogenization was accomplished by repeated vortexing and centrifuging of the PCR tubes. Libraries were prepared using the SMARTer Pico Stranded Total RNA-Seq Kit and sequenced using paired-end 2x50 base pair reads on the Illumina Hi-Seq (Study III) or Nova-Seq (Study IV) platforms.

4.7.3 Statistics, data processing, and analysis

Statistics and high-level RNA-seq analysis were primarily calculated using the R statistical computing language[184]. For single cell RNA sequencing (Study I), FastQC and QualiMap were used for quality control. Previously published protocols were used for preparation of gene counts and low-level analysis[185,186]. ccRemover[187] was used to mitigate cell-cycle-dependent effects. For bulk RNA sequencing (Studies III and IV), sequencing reads were trimmed using TrimGalore[188] and then mapped using the STAR aligner[189]. The featureCounts program was used for read assignment[190]. DESeq2 was used for transformation,

normalization and differential expression evaluation of gene counts (Studies I, III, and IV)[191]. Gene ontology analysis was performed using the geneontology.org website[192] (Study III). In Study IV, a machine-learning approach to cell deconvolution of a bulk RNA-seq sample, CIBERSORTx, was employed using a web-based tool (cibersortx.stanford.edu)[193].

5 RESULTS

5.1 ENDOTHELIAL CELL HARVESTING IN STENT RETRIEVER THROMBECTOMY (STUDY I)

We successfully induced thromboembolism and performed thrombectomy in the cervical arteries of 14 swine. Cells harvested from the stent retriever were cultured after removal of thrombotic material (n = 37 samples), and 80% of these cultures were successful. Immunohistochemistry confirmed that the cells were mostly endothelial cells. Next, we sorted cultured endothelial cells from thrombectomy and controls to a 384-well plate using FACS (Figure 5A) and performed single cell sequencing. Quality control was passed by 90% of cells. There was no significant difference in quality between ECs cultured from thrombectomy (89%) and from controls (91%). We compared cultured ECs from thrombectomy and control ECs from a surgically removed vessel. After removal of cell-cycle-dependent effects, principal component analysis showed no apparent difference between groups, and a heatmap of the 50 most variable genes did not cluster cells in different groups within or between thrombectomy cells and controls. We analyzed a selection of genes known to be involved in endothelial cell biology and found no significant differences in their expression between thrombectomy cells and controls (Figure 5B). Structural analysis of the stent retriever device by SEM showed no apparent damage or signs of corrosion.

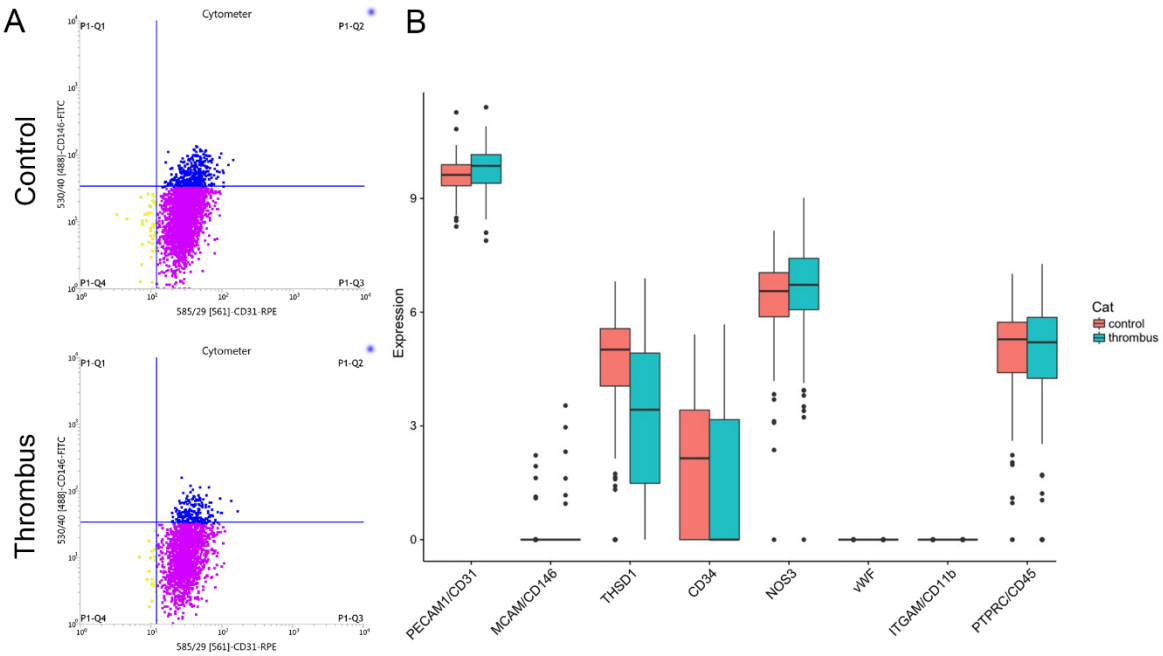

Figure 5: Transcriptome analysis of cultured endothelial cells. (A) Final fluorescence activated cell sorting gates selecting for CD31 (x axis) and CD146 (y axis) positive cells from cultures. (B) Expression of eight genes related to endothelial cell biology show similar expression between control samples and samples from thrombectomy. There were no significant differences in expression between groups.

5.2 TRANS-VESSEL WALL INJECTION (STUDY II)

We modified the trans-vessel wall device and performed injections of methylene blue in swine kidney, pancreas, and heart to demonstrate new applications of the device. The modifications to the trans-vessel wall device tip significantly decreased the force needed to

penetrate a latex membrane, from 62.9 mN to 52 mN, which was also apparent *in vivo* in terms of lower resistance to vessel wall penetration. We performed 157 injections using the newly modified version of the device. There were no persistent hemorrhages or serious adverse vascular events, despite not detaching the device tip to seal the penetration site. There was occasional small and self-limiting contrast extravasation, especially when injecting in the kidney. No contrast extravasation was seen on control angiography 5 minutes after vessel wall penetration for any injection. Transient vasospasm occurred sporadically; this is a common feature of vascular intervention in swine, and in all cases, it resolved spontaneously without the need to administer spasmolytic medication. All animals except one survived to the planned endpoints. This animal was hypotensive before the intervention began, and upon sacrifice and necropsy was found to have a restrictive cardiomyopathy. Ventricular extra beats occurred during cardiac injections, but there was no sustained arrhythmia. No myocardial infarction was seen on necropsy. For kidney injections, no signs of infarction or vascular occlusion were seen on follow up. Blood and urine biochemistry showed transient microhematuria and transient small increase in plasma creatinine. The steerable micro-catheter was useful to improve targetability of injections in the epicardium and pancreas.

5.3 MICRO-ENDOMYOCARDIAL BIOPSY (STUDIES III AND IV)

We designed and manufactured a novel endomyocardial biopsy device through iteration of some 65 design variations (Figure 6a). The device is significantly smaller than current clinical devices (Figure 6b), with a maximum diameter of approximately 0.67 mm, and therefore fits inside a micro-catheter. It is comprised mainly of superelastic nitinol and therefore highly flexible. The tissue gathering method is differs from the jaw-like forceps of the conventional devices, allowing tissue sampling depth control and a much smaller impression in the endocardial wall (about 1.5% of a conventional 7F device, Figure 6c).

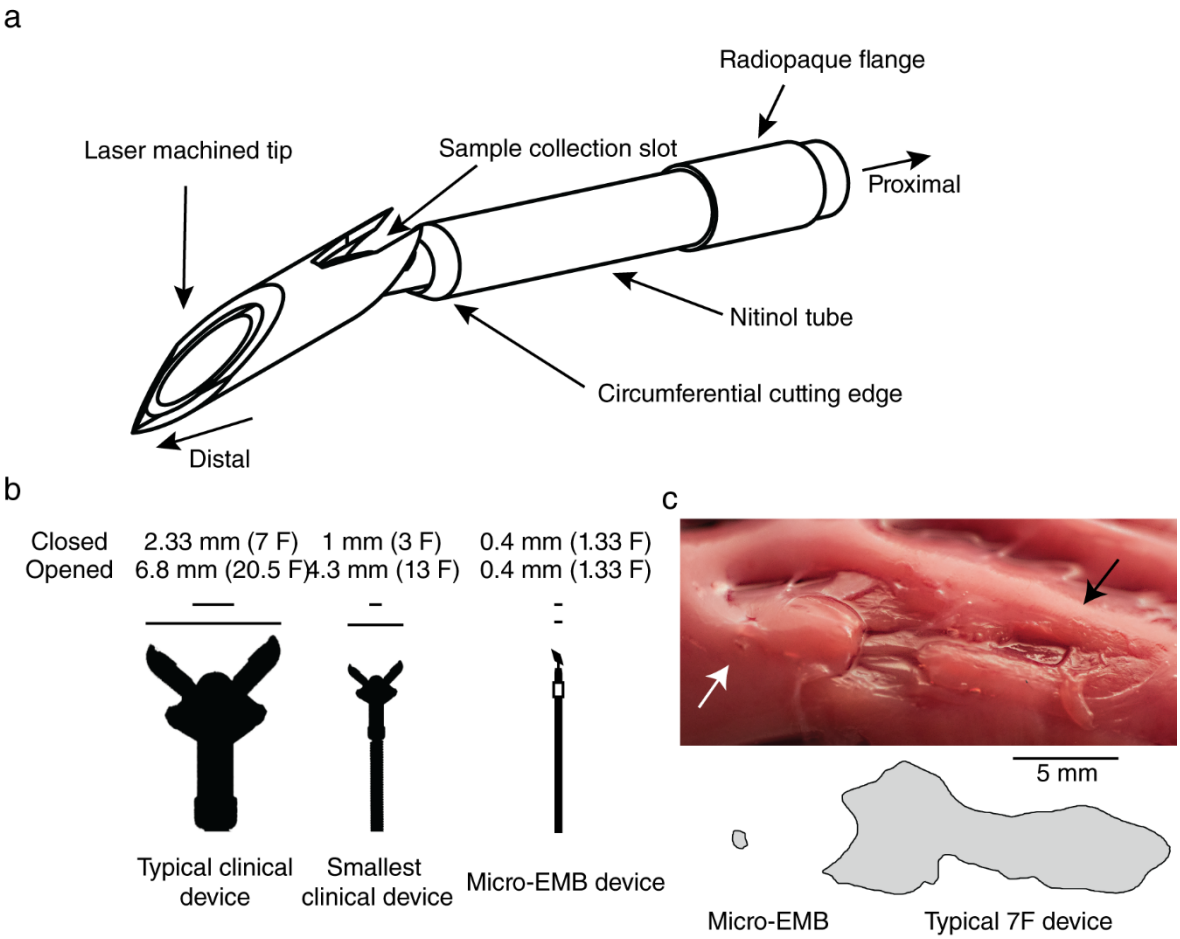

Figure 6:Micro-endomyocardial biopsy device. (a) schematic of the distal end of the device. In the proximal end, a handle enables manipulation of the tip (not shown in schematic). (b) comparison with clinically available devices. (c) comparison of endocardial lesion caused by ex vivo application of micro-EMB device (white arrow) and conventional device (black arrow).

The biopsy procedure is accomplished by first penetrating the ventricle wall, and then activating the biopsy mechanism within the myocardium by advancing and then retracting the sharpened tip. The sample is approximately 1/1000 the weight of a standard biopsy sample. This makes histological analysis difficult, as the tissue is hard to effectively section, and is significantly affected by crush artifacts. Nevertheless, histology and positive troponin I immunofluorescence showed that the tissue sampled was of myocardial origin. When using the micro-EMB device *in vivo*, we saw an 81% yield of acceptable myocardial tissue samples, visually gauged using a qualitative scale (n = 157 attempts).

5.3.1 Molecular analysis

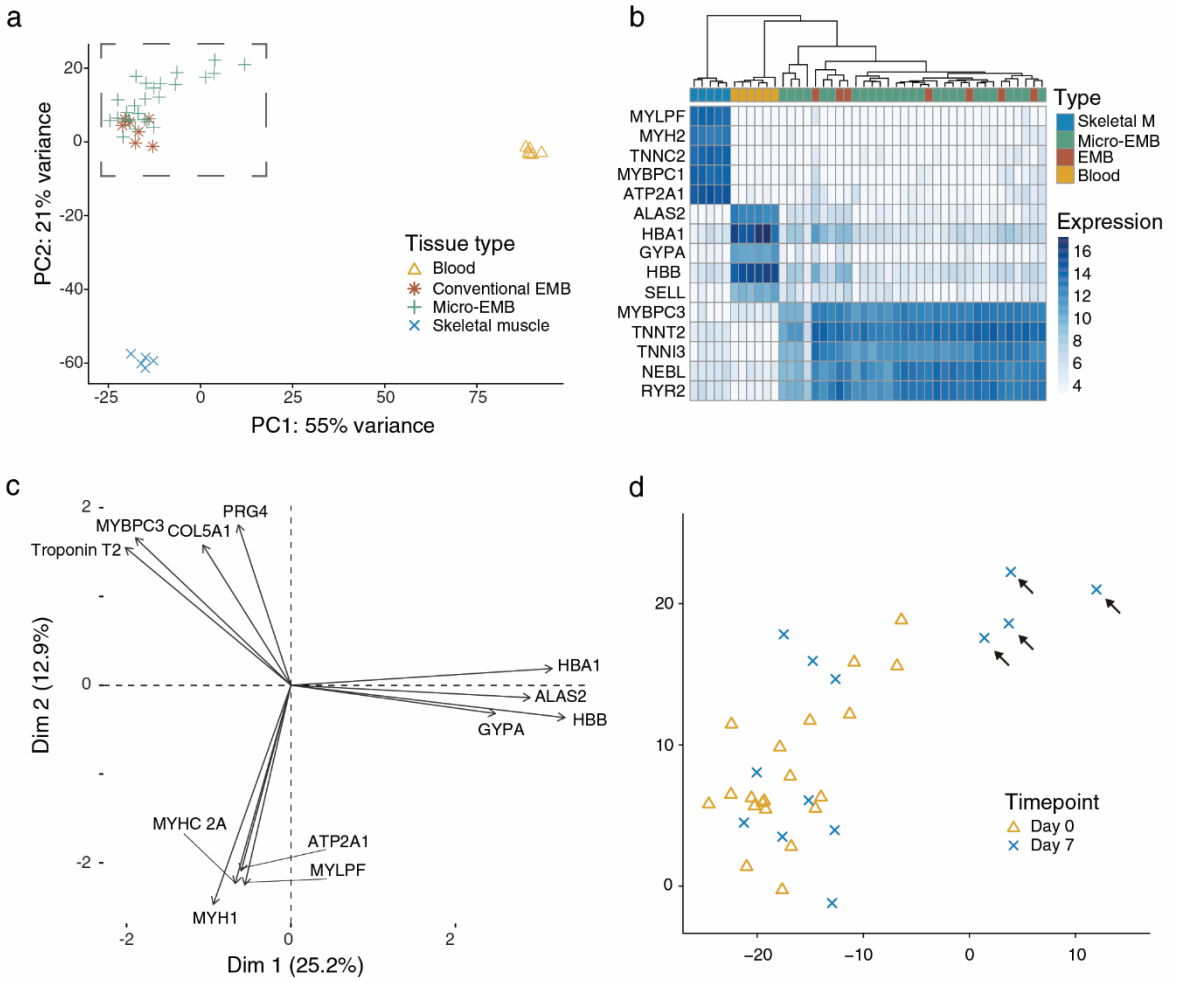

Figure 7:Transcriptomic analysis of micro-endomyocardial biopsy (micro-EMB) samples. (a) principal component analysis (PCA) of samples and controls. Micro-EMB and conventional endomyocardial biopsy (EMB) samples are grouped together. Micro-EMB samples show greater spread, indicating higher transcriptomic variance. (b) heatmap of hierarchically clustered samples showing relative expression of highly differentially expressed genes. (c) loadings of the top three principal components, showing the four genes with the highest weight of each (d) magnified view of the micro-EMB and EMB samples in (a), showing four outlier micro-EMB samples (arrows) collected on follow-up. They had upregulated genes related to "collagen fibril formation" and "wound healing".

To ensure that micro-EMB yields tissue samples that can be analyzed in a robust manner using modern omics methods, we applied RNA sequencing to samples from both healthy swine (Study III) and those affected by myocardial infarction (Study IV). First, we adapted a commercial RNA tissue isolation kit for use with the small samples yielded by micro-EMB. We then performed RNA-seq on 32 samples taken from healthy animals at two time-points (days 0 and 7) with the final design of the micro-EMB device, as well as 10 control samples using conventional devices. Additional controls from separate tissue types were also sequenced (whole blood and skeletal muscle, n = 6). We found that 91% of samples passed RNA-seq quality control. Some samples were excluded due to blood contamination (n = 7), both from the micro-EMB and conventional EMB groups. Overall, 81% of micro-EMB samples were found suitable for final analysis. Principal component analysis was used to separate samples into groups (Figure 7a, c). Micro-EMB and conventional EMB samples were grouped together, indicating transcriptome similarity, although there was a larger

variation between micro-EMB samples. These expression patterns were confirmed with hierarchical clustering (Figure 7b). Outlier micro-EMB samples (n = 4) showed an increased expression of genes related to wound healing and collagen fibril organization, possibly explaining this variation (Figure 7d). Since EMB is primarily used for rejection monitoring in heart transplantation, we examined whether RNA-seq on micro-EMB samples could reliably detect genes related to rejection. We found that the relative expression of these genes was similar between micro-EMB and conventional EMB.

In Study IV, we further investigated the possibility of finding pathological expression patterns in micro-EMB samples. AMI was induced for 1 hour and biopsies were taken before, during, and after occlusion-reperfusion. We targeted biopsy attempts to different parts of the left ventricle, with the intent to sample the ischemic region, as well as a non-ischemic area away from the affected region. We sequenced 85 samples from a mix of time-points and both areas; of these, 53 samples remained after quality control (24 samples excluded) and removal of two blood-contaminated samples. Six samples were excluded from the final analysis because of an unusually high up-regulation of collagen related genes, interpreted as being due to the samples mainly containing fibrous tissue from intramyocardial fibrous compartments.

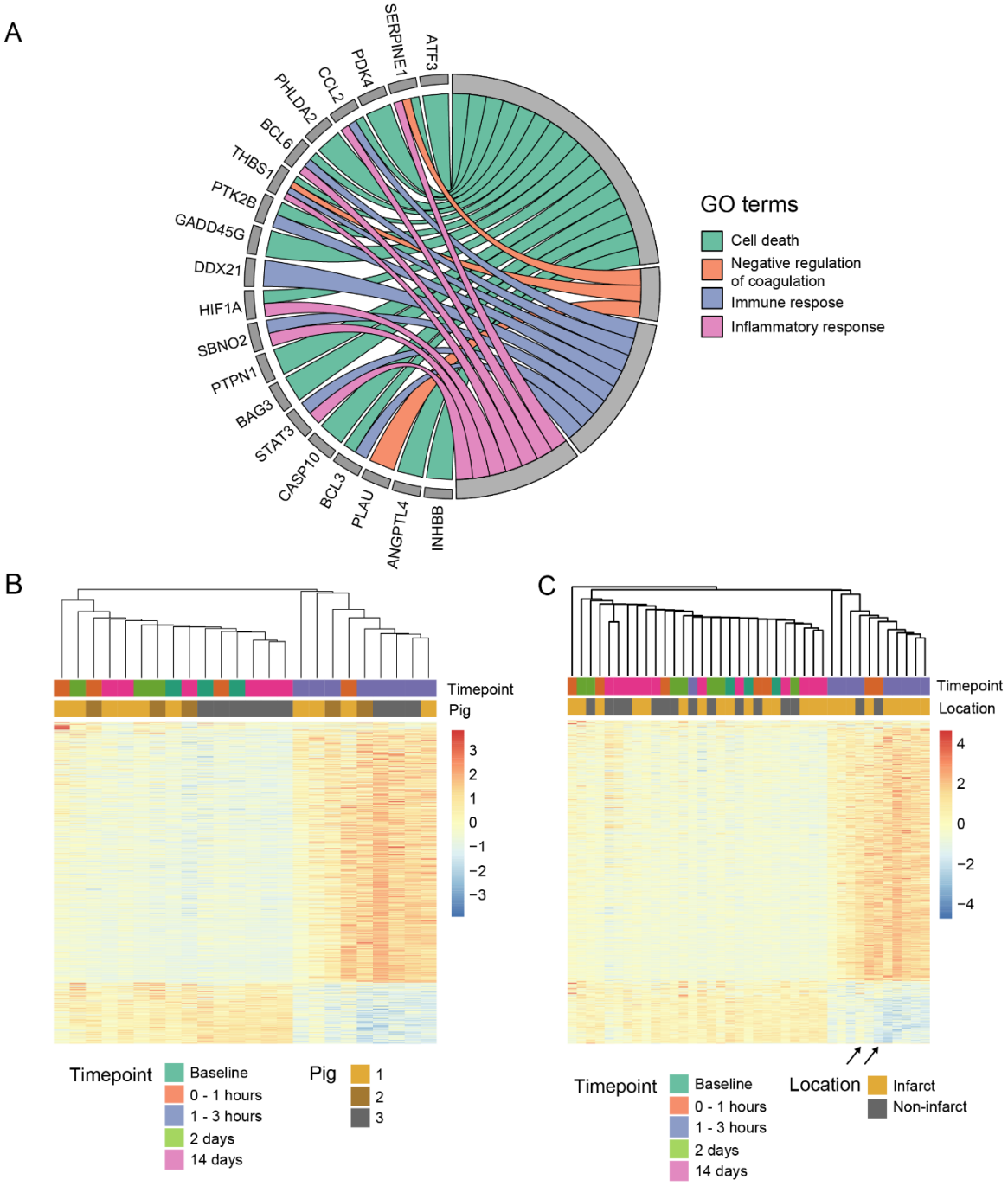

Figure 8: Transcriptome analysis of AMI samples. (A) chord plot showing regulated genes and associated biological processes. (B) heatmap showing the 1000 most regulated genes of samples from the infarct region. Hierarchical clustering has divided the samples in two clusters, separating the samples taken at 1–3 hours after occlusion from the others. (C) Heatmap the 1000 most regulated genes of samples from both infarct and non-infarct regions. At 1–3 hours, infarct samples cluster together apart from both infarct and non-infarct samples, except two non-infarct outliers (arrows).

Differential expression analysis revealed that among ischemic region samples, 1191 genes related to apoptosis, inflammation, and immune response, among others, were regulated in the short term after induction of AMI (0–3 hours). In the non-ischemic samples, only 33 genes were differentially expressed between time-points and animals, suggesting a stable tissue signal. When comparing ischemic samples to baseline (before induction of AMI), genes regulating coagulation, apoptosis, inflammatory response, and cell migration were differentially expressed at 1–3 hours and at day 2, but no clear signal was found at day 14. Conversely, non-ischemic samples showed no differential expression compared to baseline,

except in the case of a single gene at 1–3 hours, possibly attributable to noise. At later time-points, there were no significantly regulated genes compared to baseline.

We made a closer examination of potential late gene expression changes after AMI based on a deconvolution technique that allows proportions of different cell types to be estimated in a bulk RNA-seq sample[193,194]. An increased fibroblast signal and decreased signal from cardiomyocytes was seen at day 2, but at day 14 these signals were returning to baseline levels. We found no significant differences in signal from endothelial cells and macrophages.

5.3.2 Safety

In Study III, we examined the safety aspects of micro-EMB both in the device development phase (n = 23 animals) and in a longitudinal safety study (n = 3). In the development phase, only one animal died, due to inadvertent intubation of a pig bronchus. In the safety study, no serious complications occurred, and all animals survived to the pre-designated endpoint (7 days) despite a large number of biopsy attempts in each animal. Temporary arrhythmias occurred frequently in all animals, typically when a biopsy device (micro-EMB or conventional bioptome) was in contact with the endomyocardium. None of these arrhythmias required treatment other than the standard pre-treatment of each animal with amiodarone. Echocardiography showed no effect on ejection fraction and no presence of supraphysiological pericardial effusion during interventions. There were no signs of mitral or aortic valve regurgitation. On necropsy, small amounts of reddish pericardial effusion could be seen in all animals. No signs of myocardial infarction or ventricular wall rupture were seen.

We investigated the safety of micro-EMB further in Study IV, in swine with heart disease (acute myocardial infarction). Micro-EMB sampling was possible during and after AMI without any serious complications. All animals survived the induction of AMI and all biopsies, until the pre-designated endpoint (14 days). We confirmed that AMI induction had led to tissue infarction by necropsy (visible lesion) and troponin dynamics. There was no significant pericardial effusion. As in the previous micro-EMB studies in Study III, transient arrhythmias were seen, though none requiring treatment.

6 DISCUSSION

This thesis explores a variety of methods of using endovascular procedures to obtain different types of cells and tissues, and to achieve tissue access for injections of, for example, therapeutic cells or gene therapy. Since the development of endovascular intervention in the 1950s, the endovascular route has proven highly useful, and often superior, for treating various cardiovascular maladies. It can be used for superselective treatment of non-vascular diseases, such as chemoembolization of hepatocellular carcinoma, but is limited to intraluminal delivery of treatment. Endovascular techniques have been used to perform biopsy in some limited settings, most notably for myocardial disease and intravascular tumors in the large vessels. Still, the development of those established biopsy techniques has been quite stagnant, with few technical innovations and limited clinical utility. The main reasons for this are technical limitations, the high risk-to-benefit ratio, and difficulty of histopathological interpretation of small tissue samples, causing high inter-observer variability. Especially in myocardial disease, the sensitivity of endomyocardial biopsy is quite low[97,195–198], in many cases due to difficulty in targeting the area of interest in the heart. For other hard-to-reach organs, such as the brain and pancreas, no endovascular biopsy techniques exist at all. Recent developments in omics techniques allows generating large amounts of data from very small samples and opens up the possibility improving the safety of biopsy by decreasing the size of devices and tissue samples. In this thesis we propose three endovascular techniques that may solve some of the issues with endovascular cell and tissue collection, as well as further develop the trans-vessel wall technique for superselective delivery of treatment agents.

6.1 STUDY I

Study I describes a method to harvest endothelial cells from stent retriever devices used for mechanical thrombectomy in ischemic stroke. Without any technical changes to the device or modifications to the thrombectomy procedure, endothelial cells naturally adhere to the stent retriever device as it contacts the vessel wall when expanding inside the thrombus and as the device is retracted into the catheter. These cells can then be collected without damaging the device, allowing the multiple passes often performed in clinical practice. We showed that the cells could be cultured and expanded to allow extensive analysis, including by single cell RNA sequencing.

No significant differences were apparent between the cells collected in the setting of thrombectomy and the control cells. We used bioinformatic methods to try to remove any variability caused by cell cycle state, which may have masked any true phenotypic variability between cells from thrombectomy and controls, but it did not reveal any clear differences in gene expression. Since both sets of cells came from similarly sized vessels of the peripheral vasculature, this is perhaps not surprising. On the other hand, one might expect the thromboembolic occlusion of the vessel for some time (hours) to induce changes in gene expression of endothelial cells that could be detected, for example related to hemostasis or regulation of vascular tone. However, the multiple passages in cell culture are known to

change gene expression profiles, and any such acute changes induced by the thrombus are therefore likely reversed or wholly changed as the endothelial cells react to the cell culture environment. It is possible that different bioinformatic approaches such as other clustering and dimensionality reduction methods could have revealed subtle differences. Future studies may therefore change the analysis strategy, especially in human studies where a larger pathophysiological variability is expected.

When applying this endothelial cell biopsy method to the clinical setting, the cells harvested would be brain endothelial cells in patients often suffering from co-morbidities with pathophysiological implications for the intracranial vasculature, such as hypertension, diabetes, dementia, and coagulopathy. Thrombectomy-coupled endothelial cell biopsy could therefore be a promising method of obtaining endothelial cells for research. If analysis of cerebrovascular endothelial cells were to have future treatment implications in cerebrovascular disorders, thrombectomy devices could feasibly be used directly as endothelial cell biopsy devices, not only in the setting of ischemic stroke. However, the cell yield from stent retrievers in our experiments and others[199,200] have been quite low, probably necessitating cell culture before meaningful analysis is possible, unless refined harvesting and cell handling protocols for direct analysis could be developed.

6.2 STUDY II

In Study II, we further developed the trans-vessel wall technique. This technique allows exiting the vessel wall into the parenchyma of an organ after it has been reached by endovascular micro-navigation, creating a working channel to the organ through which therapeutic substrates or cells could be delivered. It has previously been studied for injections in the liver, brain, and pancreas in large animals. In this study in swine, we performed vessel exits and injections in the heart and kidney for the first time, and improved selective navigation and injection technique in the pancreas. We improved upon the design by changing the configuration of the penetrating tip, removing the detachment zone, as we found that when crossing the vessel wall in the epicardial veins and the arteries supplying the kidney and pancreas with the new device, there was no need to detach the tip to prevent bleeding. The new tip configuration reduced the penetration force required, which not only simplified the procedure but may also have cause a smaller hole in the vessel wall, contributing to the lack of significant hemorrhage seen in this study. We employed a deflectable micro-catheter to guide the trans-vessel wall device in the pancreas and heart, which markedly improved ease of navigation and selection of penetration site and penetration angle.

We detected no serious vascular or organ complications where the trans-vessel wall injections could be considered the likely cause. Although one animal died, this was likely from the anesthesia combined with a pre-existing cardiomyopathy. There were no complications in the group receiving epicardial injections with 1–3 days of follow-up, and no kidney damage was detected over a 14-day follow-up period, suggesting that the trans-vessel wall technique is applicable and safe in these organs and approaches, even without tip detachment. We have

previously shown that the trans-vessel wall technique can be used in the brain, in non-human primates, but these experiments included tip detachment. It would be of interest to attempt transvenous navigation to the brain via the dural sinuses and perform injections without tip detachment to confirm the safety of this approach in the intracranial circulation.

Using the trans-vessel wall technique without tip detachment is especially interesting in the context of biopsy, as it makes it possible to reuse a single trans-vessel wall catheter with a fitted biopsy device for multiple sampling attempts in a single session. We were not able to design such a device within the scope of this thesis, but it may now be possible to use micro-machining techniques to create a biopsy device to be used with the trans-vessel wall technique, which would enable endovascular biopsy of areas such as the subepicardium; the head, body, and tail of the pancreas; and the brain. Furthermore, the subepicardium[201,202], kidney capsule[203,204] and pancreas[205] are attractive targets for cell transplantation and gene therapy in a variety of diseases, such as heart failure and diabetes. By injecting cells directly into these sites, rather than injecting cells intravenously or intraarterially upstream, cells that are not capable of performing diapedesis can be transplanted and the engraftment rate is likely to be greater. Additionally, off-target engraftment, such as in the lungs and liver, is minimized. The improved trans-vessel wall techniques could be used as a technical platform for clinical trials of minimally invasive cell transplantation and gene therapies, although further pre-clinical studies, especially on cell and substrate retention at these sites, may be necessary first.

6.3 STUDIES III AND IV

In Study III, we developed a new endomyocardial biopsy technique, micro-EMB. This is an essentially different approach compared to the currently available devices for endomyocardial biopsy which are all based on an actuated forceps design, which grasps both endocardium and myocardium. Our device is more similar to needle biopsy in that the tissue is penetrated before the actual tissue harvesting begins. This means that the endocardium is largely bypassed, and the sample taken is mainly composed of myocardium. Since the myocardium is typically the tissue affected by the cardiac pathologies diagnosed with biopsy, this feature will likely improve diagnostic specificity of omics profiles generated from micro-EMB.

Our design is significantly smaller, has a lower profile, and fits inside a micro-catheter. The shaft portion is wholly constructed from flexible superelastic nitinol with a smaller diameter than conventional devices, improving navigability, flexibility, and deflectability. This makes it possible to more precisely guide the device using guiding catheters with shaped tips or deflectable sheaths, which can improve targeting of lesions, such as intracardiac masses and myocardial diseases with a patchy distribution of affected myocardium. In comparison, conventional EMB devices are typically stiffer and require a large deflectable sheath to navigate inside the ventricle, limiting the ability to target specific areas of the heart and avoiding damage to heart valves and chordae tendinae. With micro-EMB, intramyocardial lesions could be preprocedurally detected and mapped using cardiac MRI or nuclear imaging methods, and the map could then be fused with intraprocedural imaging guidance with

fluoroscopy[206], echocardiography, or MRI[207,208], to directly and safely target the lesions of interest.

It is also important to be able to vary the sampling site easily and safely in endomyocardial biopsy for non-focal diseases, to avoid repeated sampling of a single area. After repeated biopsy, such as in post-transplant monitoring, fibrotic scarring may develop as ventricular wall insults from the biopsies heal[209]. Sampling the fibrotic scar will not produce diagnostically valid myocardial tissue for analysis, potentially forcing yet more repeated procedures. Varying the sampling site will also increase the chance of catching diseased myocardium in patchy pathologies where the lesions cannot be seen on imaging before or during the procedure; similarly to how the prostate is systematically biopsied to increase the chance of finding prostate cancer[210]. The increased flexibility and smaller footprint of the micro-EMB device facilitate the use of radial, antecubital, and femoral access rather than jugular access, improving patient comfort and lowering operator radiation dose.

Another disadvantage of conventional EMB that is addressed by the micro-EMB technique is the size of the lesion created by the biopsy. Conventional EMB leaves a comparatively large wound in the ventricle wall that can stimulate thrombus formation[211] and thus increase the risk of thromboembolic events (stroke or pulmonary embolism). The large wound will also lead to a much larger fibrotic scar compared to micro-EMB, compounding the problem of limited targetability mentioned earlier. Finally, the wound may span the entire thickness of the ventricle wall if a biopsy is taken at a weak spot of the ventricle, particularly the right free ventricular wall, causing a perforation which may lead to tamponade and death. Conversely, the micro-EMB device creates a miniscule wound, and its penetration depth can be varied, meaning the risk of perforation should be lower. If a perforation were to occur with micro-EMB, the small hole created is less likely to cause a significant pericardial effusion or tamponade. We recently investigated the risk of tamponade when using micro-EMB and conventional EMB in a separate study, not part of this thesis, with promising results[212].

The major drawback of micro-EMB is the relative infeasibility of using conventional histopathology for the analysis of the samples. This is due to their small size and the crush artifacts created as the tissue is stored in the sample collecting slot of the device during harvesting, as well as when the tissue is removed from the device and stored. Most current diagnostics on myocardial tissue rely on histopathology, and require a relatively large biopsy specimen, to allow evaluation of the myocardial structure and whether there is infiltration of immune cells. Since several stains are typically performed, enough tissue for multiple sections is required[132]. International Society for Heart and Lung Transplantation guidelines for the histopathological diagnosis and grading of acute allograft rejection require at least four EMB tissue pieces of adequate size[132], ca. 1 mm^3. It is therefore not feasible to use conventional histopathology and grading methods on micro-EMB samples. Instead, we hypothesized that high-throughput molecular analysis methods such as genomics, transcriptomics, and proteomics would be preferred, due to their ability to extract large amounts of data from a small number of cells.

We chose to limit our initial studies to RNA sequencing, since this method is more robust and well developed than, for example, proteomics, and in comparison to DNA sequencing is more likely to yield diagnostically useful molecular signatures in myocardial disease. Study III showed that RNA sequencing was successful in a majority of biopsies, and that the tissue type sampled could reliably be distinguished from control samples of remote tissues (skeletal muscle and blood). Even so, the small size of micro-EMB tissue samples means that pathology may be missed, for example due to sampling error or too weak a molecular signal. This was partly indicated by Study III, where the variance in the gene expression pattern was greater among micro-EMB samples compared to conventional EMB. However, this variance could also represent a feature of the small, focused sample of micro-EMB. The tissue architecture of the myocardium dictates that there are likely to be local variations in the transcriptome, since some areas contain more vessels and fibroblasts while other areas are composed only of cardiomyocytes.

To address variance and detection limit as a potential weakness of the micro-EMB technique, we performed Study IV, where myocardial pathology (ischemia) was introduced in a swine model using coronary artery balloon occlusion, after which micro-EMB was performed. We showed that gene expression changes caused by myocardial ischemia could be detected by micro-EMB as early as in the first few hours after occlusion by micro-EMB. We chose acute myocardial infarction as our cardiac disease model because it is reproducible, and relatively quick and easy to induce. However, diagnosing myocardial infarction is not a primary expected use case of micro-EMB, as it is diagnosed faster and more easily using non-invasive techniques (ECG, serum troponin, patient symptoms, MRI). Rather, detection of gene expression changes in myocardial infarction was used as a proxy to demonstrate the possibility of detecting other gene expression signals associated with various myocardial diseases. Further research into specific diagnoses and associated gene expression patterns in clinical populations is needed to evolve both the field of cardiac molecular pathology and the use of micro-EMB in this context. It seems micro-EMB is sensitive enough for detection, but targeting is likely required to reduce expression profile variance.

The use of omics technologies in cardiovascular research is increasing, and there is great hope for a more personalized approach to cardiovascular medicine[213]. These methods are more expensive and more complex than conventional histopathology, and they have not yet seen widespread clinical adaptation. However, the paradigm may be shifting. Research into the molecular mechanisms behind various cardiomyopathies using transcriptomics on EMB or surgical tissue samples has yielded a number of gene expression changes[214–220] that could feasibly be targets of diagnostic and prognostic tests. Transcriptomics-based methods for detecting allograft rejection have been developed[221–227], and these methods may even be more sensitive than histopathology for detection of mild rejection[228]. Although they have been developed and validated using conventional EMB, there are some indications that analysis of smaller samples may be possible[229].

The cell yield from micro-EMB is comparable to that of fine needle aspiration, which has been successfully analyzed using microarray techniques in the setting of cancer[230–232]. Myocardial tissue is different from cancer tissue and has properties that can make RNA isolation difficult. Nevertheless, we showed in Study III that an adapted commercial RNA isolation kit could be used with consistent results. Clinical EMB usually involves taking a minimum of 5 biopsies in each session, and sometimes up to 10, to ensure enough representative tissue material for diagnosis is gathered. It is not yet clear how many micro-EMB samples would be needed for confident diagnosis of, for example, transplant rejection. Since each individual pass of micro-EMB is likely to present a smaller risk than EMB and cause less damage to the ventricular wall, it should be acceptable to routinely take more than 5 biopsies. The recommended number of passes to achieve an acceptable chance of reaching diagnosis should be evaluated in clinical trials, but our studies indicate that 5–10 samples will likely be an acceptable target.

6.4 STRENGTHS, LIMITATIONS, AND METHODOLOGICAL CONSIDERATIONS

Strengths of this thesis include a variety of different approaches for different target organs and tissues for transvascular access and biopsy, the use of large animals to mimic the clinical situation more closely, and the development of a complete workflow in endovascular biopsy, from interventional procedure to omics analysis. The development of these endovascular devices and techniques was facilitated by the design of our laboratory and our collaboration with the Royal Institute of Technology in Stockholm, which allowed us to control and iterate throughout the entire process including device design, prototyping, manufacturing, *ex vivo* simulation, and *in vivo* large animal studies in a clinical grade interventional laboratory. We were able to study both the technical feasibility and the apparent safety of the procedures, monitoring for complications both in the immediate term and the short term, with up to 14 days follow-up. We evaluated safety both intra- and periprocedurally as well as post-mortem, using invasive and noninvasive diagnostic techniques, such as angiography and ECG monitoring, as well as gross pathology evaluation of organs in which biopsies and injections had been performed. We performed repeated injections or biopsies in all organs and tissues investigated, yielding a relatively large number of passes. In the clinical situation, far fewer injections or biopsies are likely to be performed in each patient. This illustrates the safety of the procedures beyond the minimum passes likely needed for clinical effectiveness.

This thesis has several limitations. As we focused our efforts on technical development and validation of the devices described, we did not design the studies to definitively demonstrate the safety of each device and technique. In Study I, we performed mechanical thrombectomy according to clinical routine, without any technical or practical changes to the procedure. Mechanical thrombectomy has been demonstrated to be relatively safe, or at least to have an acceptable risk-to-benefit ratio in the treatment of ischemic stroke. It is highly unlikely that the cell harvesting procedure would affect the safety of the thrombectomy procedure,

especially as we found no effect on the stent retriever device after dissociation buffer submersion and washing in saline.

Studies II – IV concerned novel techniques developed in-house, for which safety evaluation is a major concern. Although we found no clinically relevant complications related to the procedures in the relatively large number of animals studied, detecting low frequency complications, such as cardiac tamponade during EMB and significant hemorrhage during trans-vessel wall injections, would require a prohibitively large number of animals in each study. The animals were also healthy, except in Study IV, where a small myocardial infarction was induced to evaluate the safety of micro-EMB in a diseased heart. In the clinical situation, all patients in need of any of the techniques described in this thesis are likely to suffer from severe disease in the organ of interest, and may also have systemic disease that affects the safety equation, such as coagulopathy or kidney failure.

We studied the safety of micro-EMB in acute myocardial infarction. However, the infarctions created were relatively small, as we occluded the LAD beyond the third diagonal arteries. Swine are known to be very sensitive to ischemia with regard to arrhythmia and death[233,234], and more proximal occlusion would probably have caused significant mortality in the study group. Moreover, we needed a relatively small infarction in order to be able to consistently sample from "unaffected" myocardium, which would have been difficult if a large area in the ventricle was ischemic. Coagulopathy also presents a special case, as hemorrhage is the most likely complication of the trans-vessel wall technique and as swine are considered hypercoagulable compared to humans[235]. We used catheter angiography to evaluate any post-intervention hemorrhage, rather than, for example, CT angiography. Some studies indicate that CT angiography may have a greater sensitivity for small active hemorrhage, as compared to catheter angiography[236], although there is no conclusive evidence comparing superselective catheter angiography to CT angiography. Studies II – IV may therefore have underestimated the clinical risk of hemorrhage of the techniques studied. We chose catheter angiography as the detection method because it was technically infeasible to perform CT angiography after each intervention, and we consider the sensitivity to be sufficient to detect any clinically significant hemorrhage.

We also did not perform histopathological evaluation of the organs used for injections or biopsies, meaning subclinical microscopic injury may have been missed. True evaluations of technique safety can only be performed in clinical trials. Nevertheless, studies in large animals such as the ones undertaken in this thesis work are necessary to demonstrate the feasibility and apparent safety of a new device or technique before clinical trials are undertaken, in the interest of the safety of human study participants[237,238].

In Study II, we performed injections only of iodine contrast and methylene blue, to validate the correct localization and spread of injected material. We did not directly evaluate stem cell transplantation or gene therapy. We considered effectiveness of cell engraftment and gene therapeutic transfection to be best studied in specific treatment studies in animal disease

models. We chose not to perform follow-up experiments for pancreas injections, as this has been studied previously[48].

Furthermore, we did not design the studies using RNA sequencing to detect gene expression patterns of clinically relevant diseases. Therefore, we cannot yet confidently claim that stent retriever cell harvesting or micro-EMB coupled to RNA sequencing can detect pathophysiologically relevant gene expression signals or accurately diagnose disease in a clinical setting. We have, however, shown indirect evidence that this should be possible, considering the overall robustness of our sample isolation and RNA sequencing protocols and the ability of micro-EMB to detect gene expression changes in myocardial infarction. Others have shown that similar amounts of sample material can be sufficient for molecular diagnosis in other fields. Further studies in patients with relevant diseases are needed to confirm this important underpinning of endovascular micro-biopsy and cell harvesting.

"Omics" encompasses more than the transcriptomic techniques explored on our samples, particularly genome sequencing (DNA-seq), epigenomics, proteomics, and metabolomics. As mentioned previously, we chose to focus on RNA sequencing because we considered the transcriptome more relevant to endothelial cells and myocardial tissue than genomics, as the cells are unlikely to contain important genomic alterations. We also chose not to explore proteomics and metabolomics on the biopsy samples; these, can be considered less mature technologies[166], and thus harder to implement in this initial technical feasibility study. Most proteomics techniques have typically been studied on larger cell and tissue samples than those produced by stent retriever cell harvesting and micro-EMB, since proteins cannot currently easily be amplified like DNA and RNA to increase the signal[239,240]. It is therefore important to perform further pre-clinical studies to confirm the possibility of analyzing micro-EMB samples and harvested endothelial cells with other omics techniques before they are attempted in clinical trials.

7 CONCLUSIONS

The studies in this thesis explored a variety of novel approaches using endovascular techniques to access organs and tissues that are difficult to selectively and safely access with other means, and to perform biopsy on some of these organs and tissues. Superselective tissue access and biopsy as a concept requires a multitude of different approaches, each tailored to the target organ or tissue of interest. No single device will be appropriate for all situations, and it is crucial that clinical trials of endovascular biopsy identify and optimize the best approach and device design for each separate indication.

In some cases, off-label use of clinical devices used for treatments (stent retriever, guidewires and coils) can be used to biopsy endothelial cells. We showed that endothelial cells could be harvested from stent retrievers when performing thrombectomy, and that the cells could be expanded in culture. This could be used to routinely gather endothelial cells from patients undergoing endovascular stroke treatment. Cell cultures could be stored to create a biobank of intracranial endothelial cells, enabling new approaches in vascular research. Since stent retrievers and similar endovascular instruments are they are not specifically designed for endothelial cell sampling, a more optimized approach may be achievable using dedicated devices. A useful goal of a dedicated device could be increasing endothelial cell yield from each harvesting pass, especially if one is intending to use endothelial cell biopsy in clinical diagnostics.

Some organ sites are especially hard to selectively reach and may hold great potential for targeted cell transplantation or gene therapy. Our improvements to the trans-vessel wall technique have increased its safety and ease-of-use, and we have now demonstrated the feasibility of new targets in the heart, kidney, and pancreas for injections or biopsy with potential new devices fitted to the trans-vessel wall device.

Technical developments in minimally invasive endovascular materials have enabled us to design a novel endomyocardial biopsy device which may have superior operational characteristics to conventional devices, especially for the repeated biopsy required when monitoring of cardiac transplants and for the use of molecular analysis methods to improve invasive cardiac diagnostics.

Endothelial cell sampling and micro-EMB rely on low-input high-throughput molecular analysis methods, which are suitable for mechanistic research in human subjects but need to be developed further for use as diagnostic instruments. Overall, there is an exciting potential in using endovascular techniques both for superselective tissue biopsy and to deliver intra-tissue agents in the modern era of personalized medicine. Coupled with developments in molecular analysis methods as well as cell-based and gene-based treatments, endovascular tissue access and biopsy may provide solutions to pressing problems in clinical medicine, provided that clinical translation is successful.

8 POINTS OF PERSPECTIVE

For clinical diagnostics, biopsy with histopathology remains the gold standard method to interrogate tissue for disease. Our understanding of the pathophysiology of disease has evolved rapidly, and morphological analysis of diseased tissue is now known to be far from sufficient to accurately describe most disease panoramas, leading to the prevalent use of omics methods in research. As pathology enters to the digital molecular age, we will probably move past histopathology as the gold standard for diagnosis and towards an integrated approach where diseases are defined by a combination of physiological parameters, imaging, and molecular profiles of tissue. To facilitate this paradigm shift in diagnostics, access to pathological tissue suitable for omics analysis is crucial. It is therefore necessary to improve methods of biopsy, in order to strengthen investigation into organ and disease phenotypes in human patients while maintaining safety in an era of less invasive medicine.

Biopsy should ideally be accurate, minimally invasive, safe, cheap, and simple to perform. However, as disease complexity and overall patient morbidity increase, finding methods that satisfy all criteria becomes more challenging. This thesis presents a variety of approaches in this regard. There is no reasonable alternative to intracranial endothelial cell biopsy, and so the risks and costs of stent retriever cell harvesting could therefore be motivated. This thesis also presents methods that seek to improve on current techniques, by increasing accuracy and safety, while being more complex and in some cases more expensive than alternative methods. The trans-vessel wall technique could potentially be used to deliver a biopsy device, which would be useful in cases where direct puncture of organs such as the pancreas cannot safely be performed. If the trans-vessel wall technique could be used to biopsy the brain without the need for invasive craniotomy, this could be both more cost-effective and safer than stereotactic biopsy. Micro-EMB is likely to be relatively cost-neutral compared to traditional EMB, if the same analysis methods are used; that is, the main driver of cost is omics analysis. However, omics methods are steadily decreasing in cost[138,139], and in comparison to conventional histopathology they offer potential advantages of decreased inter-rater variability and large-scale automated analysis.

Techniques of cell and RNA isolation are also swiftly improving, especially in the field of single cell RNA sequencing[241]. The data volumes and analytical complexity are even larger in single cell RNA sequencing compared to bulk RNA sequencing, but the potential upside of single cell resolution makes this method highly useful in identifying variation in gene expression signatures of normal cells, describing new cell subpopulations, and distinguishing pathological patterns of gene expression. Single cell RNA sequencing has recently been achieved on the adult human heart[242], which may make it possible to perform single cell sequencing on cardiomyocytes and other cells harvested during micro-EMB. Indeed, a highly promising use case of micro-EMB is for large scale research on patients with cardiomyopathies or unexplained heart failure where, although both conventional and micro-EMB are unlikely to be diagnostic, analysis of myocardial tissue with omics techniques may lead to new discoveries of mechanisms and biomarkers behind heart failure. A favorable

safety profile and ease-of-use in the left ventricle may mean left ventricular micro-EMB could be become part of the trans-radial diagnostic coronary angiography procedure routinely performed in the clinical investigation of unexplained heart failure, with greater acceptance by clinicians, patients, and institutional review boards compared to conventional EMB.

The evolution of modern personalized medicine depends on the identification of the unique genotype and phenotype combination presented by the individual patient and their disease. By finding this signature, precision treatments can be developed that are highly tailored to the unique combination of patient and disease characteristics, improving health outcomes. Diagnoses will evolve more complex taxonomy, based on a greater understanding of the pathophysiological mechanisms behind diseases. This which will likely lead to the development of more truly etiological treatments to be implemented in clinical medicine. Proper diagnosis in this context will include investigation into global patient genotype (e.g. DNA sequencing) and local genotype and phenotype (e.g. diseased tissue and pathogen DNA, RNA, and protein sequencing, tissue imaging in vivo and ex vivo, physiological measurements). Local dynamic genotypic and phenotypic evolution is also likely to be important in increasing diagnostic specificity, especially in cancer and infectious diseases[146,243,244].

Omics methods allow analysis of tissue and cell states at an unprecedented scale and speed. Large amounts of data are generated, and there is intense research into the optimal bioinformatic methods to analyze and gain new insights from omics data. Multi-omics approaches in which different types of molecules (DNA, epigenetic alterations, RNA, proteins, metabolites, microbiome) are analyzed in a single biopsy are likely to become more common in the investigation of the mechanisms behind and molecular evolution of human pathology[137]. Cost and complexity may be important reasons why these novel high-throughput analysis methods have yet to be developed into clinical tests that can reach widespread adoption[157]. There is considerable difficulty in creating a standardized diagnostic or prognostic test that can be used across laboratories and hospitals to find true pathological signals among technical and biological noise. Clinical tests using omics methods will likely depend on centralized technical processing, sequencing, and large-scale cloud-based data analysis using machine learning. Centralization of sample processing and sequencing to dedicated omics facilities, such as the National Genomics Infrastructure in Sweden[245], will improve standardization, lower costs, and increase speed of analysis as large amounts of samples from a variety of hospitals and institutions are analyzed *en masse*.

Clinical translation is the most important challenge of novel medical technologies. While studies in large animals offer a comparatively solid ground on which to base initial clinical trials, there remain practical hurdles to overcome. Adapting the devices and techniques presented in this thesis to the human clinical situation will make certain elements simpler, while others may become more complex. For example, the human vascular system is generally easier to navigate than that of swine, particularly the veins in our experience, which should make transvenous epicardial, intracranial and kidney navigation easier. The pig heart

is also more susceptible to arrhythmia than the hearts of other mammals[246–248], which should simplify micro-EMB and cardiac access using the trans-vessel wall technique. On the other hand, human hemostasis differs from that of swine, and humans may be more prone to hemorrhage, especially patients with coagulopathy[235]. It is therefore on balance still unclear if the techniques presented here will successfully translate to clinical practice. Alterations to the devices or alternative approaches may be required to achieve success. The studies in large animals that make up this thesis do, however, supply a solid ground on which to base further research, both pre-clinical and clinical. There is a need in both research and clinical medicine for new or improved approaches to minimally invasive tissue access and biopsy, and hopefully this thesis contributes the development and future clinical integration of such approaches.

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