Chapter 6: Summary
The present thesis describes several clinical studies on the topic of intravenous myocardial contrast echocardiography, and an experimental study on the effect of ultrasound contrast on the cellular level. **Chapter 1.** describes ultrasound contrast agents and their unique properties that make them useful for ultrasound imaging and therapeutic purposes. Ultrasound contrast exists from microbubbles that can pass the lung and are relatively stable in blood. They produce non-linear resonance under influence of ultrasound, which enables selective imaging of ultrasound contrast, and is the basis of contrast-enhanced imaging. With ultrasound of high intensity, microbubbles collapse, thereby causing microstreaming, shock waves, motion of fluid, and increased cell permeability. Microbubbles can function as carriers for drugs and genes. Using an intravenous injection of microbubbles with local application of ultrasound, drugs or genes can be released locally. Several studies demonstrated that microbubbles enhance gene uptake and expression. A second, intriguing issue is the development of targeted microbubbles. Biochemical properties of microbubbles create the possibility to incorporate ligands into the microbubble shell. Targeted microbubbles may selectively bind to specific cell types, inflamed tissue or activated endothelium, with the possibility of local treatment. A third field of interest is sonothrombolysis. Recent studies showed that the forceful collapse of microbubbles enhances lysis of blood clots. As a large part of cardiovascular diseases is caused by thrombotic disease, thrombolysis may become an important future technique for non-invasive recanalization of acutely occluded vessels, of which the first clinical studies have already proven the benefit.

**Chapter 2.** of this thesis contains a study on the quantification of myocardial perfusion. Previous studies demonstrated that, according to a model designed by Wei et al., a continuous infusion of ultrasound contrast allows absolute quantification of myocardial perfusion in an experimental and in vivo setting. We aimed to validate this technique in humans and compare myocardial contrast echocardiography (MCE) with the gold standard, positron emission tomography (PET). Sixteen healthy volunteers underwent rest and adenosine real-time MCE. Replenishment curves were calculated from end-systolic images and fitted to an exponential equation \( y = A(1 - e^{-\beta t}) \). The average myocardial blood flow per patient, estimated as the product of \( A \) and \( \beta (P_{E\text{corr}}) \), significantly increased from 1.43±0.35 IU s\(^{-1}\) at rest to 5.70±2.44 IU s\(^{-1}\) at stress (\( P<0.01 \)) with MCE, resulting in a mean flow reserve of 3.94±1.31. \( P_{E\text{corr}} \) correlated significantly with MBF\(_{\text{corr}}\). This correlation was found in all territories (left anterior descending artery: \( r=0.87, P<0.01 \); right coronary artery: \( r=0.66, P<0.01 \); circumflexal artery: \( r=0.75, P<0.01 \)). At rest only, no significant relations were demonstrated between PET and
MCE. Heterogeneity was significantly larger for MCE (coefficient of variation 32±15%) than for PET (9±6%) measurements (P<0.01). The results of the present study indicate that, especially at stress, quantification of global MBF correlates well with PET. Accurate quantification of rest MBF and coronary flow reserve is more challenging.

Chapter 3.1. provides a discussion about the safety of the use of contrast agents. The introduction of ultrasound contrast agents has led to a markable improvement of diagnostic capabilities in echocardiography. The occurrence of fatal adverse events, that were reported in a post marketing analysis of more than 150,000 studies of Sonovue® (0.002%), led to a temporary withdrawal, and the addition of several contra-indications for the use of this ultrasound contrast agent, although a causal relationship between the fatal cases and the use of Sonovue is debatable. The risk associated with the use of this ultrasound contrast agent should be judged carefully, taking into consideration the prevalence of adverse effects of other contrast media and diagnostic procedures used in cardiology. Large studies showed that fatal adverse events occurred in 0.0004% with GTPA (causal relation not clear), 0.0006% with ionic and non-ionic contrast media, and 0% in radiopharmaceuticals. When compared with exercise testing (0.005%) and dobutamine stress echocardiography (which has a relatively high serious adverse event rate of 0.43%), that have comparable values for assessment of coronary artery disease, administration of UCAs seems to be relatively safe. This manuscript stimulates the discussion about which risk is clinically acceptable for the diagnostic value of a specific test.

In Chapter 3.2. a retrospective study is described. Previous studies suggested that during MCE, premature ventricular complexes (PVCs) occurred that were caused by a ‘flash’ of high intensity ultrasound, which is applied for destruction of microbubbles. As safety is an important issue for registration of ultrasound contrast for myocardial perfusion imaging, we aimed to assess the occurrence of PVCs during real-time rest and adenosine MCE in a retrospective study in fifty healthy volunteers and twenty-six patients with stable coronary artery disease. The number of premature contractions was counted before contrast infusion, at rest, and at adenosine stress. Occasional premature contractions occurred following a flash. In healthy subjects, the occurrence of PVCs at baseline (0.04±0.23 PVCs/min) was similar at rest (0.04±0.23 PVCs/min, P=NS), and adenosine stress (0.03±0.14, P=NS). In CAD patients, the occurrence of PVCs at baseline was 0.30±0.76 PVC/min, compared to 0.29±0.74 at rest (P=NS), and 0.34±0.74 during adenosine stress (P=NS). The number of subjects
demonstrating PVCs did not increase during MCE. The occurrence of premature atrial complexes during MCE was not increased compared to baseline in any of the study groups. We conclude that real-time MCE is safe with respect to the occurrence of premature contractions and arrhythmias.

Chapter 4.1 reviews the current status of intravenous MCE. During the last 10 years, many studies have assessed the value of MCE for myocardial perfusion imaging. Several imaging modalities have been developed that make use of the non-linear resonance of microbubbles during insonification. These modalities can be divided in high-power and low-power, allowing triggered and real-time imaging (for simultaneous assessment of wall motion and perfusion) respectively. Both techniques can be used for (semi)-quantification of myocardial perfusion, whereas in humans only real-time MCE has been demonstrated to correlate well with the gold standard for assessment of myocardial perfusion, positron emission tomography. In patients with stable coronary artery disease, detection of significant coronary artery disease is currently performed with dobutamine stress echocardiography, and cardiac scintigraphy. Several studies show that the agreement of MCE with these contemporary techniques is high (65-92%). In addition, comparison of the latter techniques and MCE for detection of significant coronary artery disease with coronary angiography as gold standard, shows that the sensitivity of MCE is significantly higher compared with DSE/SPECT (85% versus 71%, p<0.001), with a similar specificity (74% versus 71% respectively, p=ns). Furthermore, in patients with unstable ischemic heart disease, MCE appears to be a sensitive tool for detection of impaired myocardial perfusion (82%), and thus can play an important role in the diagnosis of acute coronary syndromes. In addition, in patients after acute myocardial infarction, MCE accurately defines areas of (no)-reflow, and the presence of adequate reperfusion, which is a predictor of functional recovery (presence of contrast predicts recovery with a sensitivity of 82% at follow up), and death. Besides diagnostic value, MCE thus has important prognostic value in the setting of acute ischemic heart disease. This meta-analysis emphasizes the potential of MCE for routine clinical use.

Chapter 4.2. Previous studies assessed prediction of recovery of segmental function after acute myocardial infarction with rest MCE. As vasodilator stress may improve detection of collateral vasculature and residual intact microvascular function, we aimed to assess the value of adenosine stress MCE for prediction of functional recovery, and for estimating infarct size. 25 patients underwent rest and adenosine real-time MCE in the subacute phase after
myocardial infarction. Residual perfusion as measured with rest and stress MCE correlated significantly with residual ST-elevation after reperfusion ($r=0.86$, $p<0.01$, versus $r=0.66$, $p<0.01$ respectively), and peak CK-MB ($r=0.76$, $p<0.01$ versus $r=0.85$, $p<0.01$ respectively). The sensitivity for prediction of recovery was significantly higher with adenosine stress (97% versus 88%), with a similar specificity. All patients tolerated adenosine MCE well. We conclude that adenosine stress MCE is a safe and clinically feasible tool for prediction of recovery of function in the subacute phase after AMI, with additional value compared with rest MCE.

Chapter 5.1. In the present study, we addressed the interactions between ultrasound, microbubbles and living cells as well as consequent arising bioeffects. We specifically investigated whether hydrogen peroxide (H$_2$O$_2$) is involved in transient permeabilization of cell membranes in vitro after ultrasound exposure at low diagnostic power, in the presence of stable oscillating microbubbles, by measuring the generation of H$_2$O$_2$ and Ca$^{2+}$ influx. Ultrasound, in the absence or presence of SonoVue$^\text{TM}$ microbubbles, was applied to H9c2 cells at 1.8 MHz with a mechanical index (MI) of 0.1 or MI 0.5 during ten seconds. This was repeated every minute for five times. The production of H$_2$O$_2$ was measured intracellularly with CM-H$_2$DCFDA. Cell membrane permeability was assessed by measuring real-time changes in intracellular Ca$^{2+}$ with Fluo-4 using live-cell fluorescence microscopy. Ultrasound, in the presence of microbubbles, caused a significant increase in intracellular H$_2$O$_2$ at MI 0.1 of 50% and at MI 0.5 of 110% compared to control ($p<0.001$). Furthermore, intracellular Ca$^{2+}$ increases at both MI 0.1 and 0.5 in the presence of microbubbles, which was not detected in the absence of extracellular Ca$^{2+}$. In addition, in the presence of catalase Ca$^{2+}$ influx immediately after ultrasound exposure was completely blocked at MI 0.1 ($p<0.01$) and reduced by 50% at MI 0.5 ($p<0.001$). Finally, cell viability was not significantly affected, not even 24 hours later. These results implicate a role for H$_2$O$_2$ in transient permeabilization of cell membranes induced by ultrasound-exposed microbubbles.

Chapter 5.2. Although gene therapy has great potential as treatment for diseases, clinical trials are slowed down by the development of a safe and efficient gene delivery system. This part of the thesis reviews the viral and non-viral vehicles used for drug and gene delivery, and the different delivery techniques, thereby focusing on the delivery through ultrasound contrast agents. Currently, the mostly used viral vectors include retroviral-, lentiviral-, adenoviral-, and adeno-associated viral vectors. Non-viral gene delivery techniques include micro-injection, the gene-gun, magnetofection, micelles, liposomes, and electroporation. The current
techniques mostly lack efficiency, whereas safety of viral vectors is doubtful. The development of ultrasound contrast agents, containing encapsulated microbubbles, has increased the possibilities for therapy. Microbubbles are safe, inert intravascular tracers. Their chemical composition allows binding or incorporating drugs, genes, and ligands in the bubble or the bubble shell. Ligands can be used to target the microbubbles to specific tissue. Destruction of the bubbles by ultrasound will result in local release of their contents, whereas ligands can be used to target the microbubbles to specific tissue. The results of several studies provide persuasive evidence, that a future role of microbubbles for gene delivery is promising.

**Future perspectives in myocardial contrast echocardiography**

As described in this thesis, many studies already have investigated the value of myocardial contrast echocardiography for clinical use in patients with coronary heart disease. Up to now, all these studies were performed as part of medical research, as a contrast agent that is registrated for myocardial perfusion imaging is still lacking. In the near future, contrast agents will become available for perfusion imaging in routine clinical care. Implementation of MCE requires availability of ultrasound equipment, and training of personnel. Provided these conditions are fullfilled, MCE probably has potential to compete with contemporary techniques for assessment of coronary artery disease. Recent developments in three-dimensional echocardiography have simplified image acquisition with use of a 3-dimensional matrix transducer. These transducers produce a homogeneous ultrasound field, which can increase image quality further. Although 3D-left ventricular opacification can be performed with the current ultrasound equipment, technical limitations preclude real-time imaging of myocardial perfusion in three dimensions. If these limitations can be addressed adequately, 3D echocardiography has the potential to provide a simple and fast technique for assessment of perfusion.

**Future perspectives for therapeutic contrast echocardiography**

Future therapeutic applications of ultrasound contrast agents, can be grossly divided into two areas: the area of drug- and gene delivery, and the area of contrast enhanced thrombolysis. As the genetic basis of diseases is increasingly understood, gene delivery will gain importance in the near future. Many experimental animal studies show that microbubbles enhance delivery of genes. Although the results of these studies leave no doubt about the effectivity of microbubbles, the working mechanism remains unresolved. Future research should focus on assessment of the mechanism of gene delivery with microbubbles on the cellular level, the
effect of variation of ultrasound parameters such as frequency, intensity and duration, and on
the specific type of microbubbles. Unraveling the mechanism will enable us to potentiate the
effect of contrast-enhanced gene delivery, which is an fundamental condition, before this
technique can be used in clinical studies.

The second important area of interest is sonothrombolysis. In the Western society, thrombo-
occlusive diseases are responsible for the majority of disease-related mortality. Experimental
studies showed that insonified ultrasound contrast has thrombolytic effect, both in vitro and in vivo. Recently, in patients with acute stroke, microbubbles were succesfully used as adjuvant
therapy with thrombolysis. Although the majority of patients with acute myocardial infarction
is currently treated with a percutaneous coronary intervention, many patients abroad still
receive thrombolysis. In this patient group, adjuvant in-hospital therapy with contrast agents
and ultrasound application could possibly speed up recanalization.

According to the adage ‘time is muscle’, the speed of recanalization is an important predictor
of outcome, also for patients with acute myocardial infarction that are treated with PCI. Recanalization of the occluded artery should begin before arrival of the patient in the hospital.
With use of dedicated equipment, sonothrombolysis could provide a non-invasive, fast
technique for timely restoration of the occluded vessel.

As currently no patient-based studies on this topic have been performed, future research will
need to focus on the effectivity of microbubble-enhanced sonothrombolysis in humans.