TARGET IDENTIFICATION AND CHARACTERIZATION IN TRANSGENIC CARDIOVASCULAR DISEASE MODELS OF ATHEROSCLEROSIS AND HEART FAILURE

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1. Abstract of scientific results

Myocardial infarction and ensuing heart failure are leading causes of death worldwide with limited treatment options. The major aim of my thesis was to identify new pathways involved in the pathogenesis of these diseases, which could be exploited as possible drug targets in the future.

Atherosclerosis is the major cause for coronary artery disease and myocardial infarction. Current therapeutic strategies target the atherosclerosis-promoting lipid profile, mostly low-density lipoprotein (LDL) cholesterol. In search for possible new therapeutic approaches, I focused on inhibition of the angiotensin-converting enzyme. In addition to the established beneficial profile of angiotensin-converting enzyme (ACE) inhibitors in hypertensive patients, recent data indicate that this class of drugs could also interfere with pathomechanisms of atherosclerosis without targeting lipids, but underlying mechanisms are less clear.

The first project of my thesis focused on the identification of atheroprotective mechanisms induced by treatment with the prototypic ACE inhibitor, captopril, in ApoE-deficient mice as an experimental model of atherosclerosis. Beneficial effects of ACE inhibition are mostly attributed to inhibition of angiotensin II-mediated AT1 receptor activation, which promotes the generation of reactive oxygen species (ROS) and thereby could aggravate atherosclerotic plaque formation. To differentiate between ROS-dependent and ROS-independent therapeutic effects of ACE inhibition, I performed a comparative study between the ACE inhibitor captopril and the antioxidant vitamin E. Treatment effects were monitored on a whole genome basis by microarray gene expression profiling and by quantitation of atherosclerotic lesion size in the aorta.

My study showed that captopril treatment led to a stronger reduction of atherosclerotic plaque area in the aorta by 88.1 ± 7.5 % compared to vitamin E, which reduced the lesion area by 45.8 ± 11.5 %. While more than 30 % of vitamin E-regulated probes were concordantly regulated with captopril, only 14 % of captopril-regulated genes showed concordant regulation with vitamin E. Among concordantly regulated genes, gene ontology analysis indicated that the antioxidant capacity of captopril and vitamin E counteracted the proliferative and synthetic phenotype of vascular smooth muscle cells. In contrast, captopril, but not vitamin E, reduced the migration of pro-inflammatory and pro-atherogenic monocytes and T-lymphocytes into the aorta. In addition, only captopril was able to prevent the atherosclerosis-induced down-regulation of 30 perivascular nerve-specific genes, a treatment effect that was not recognized before with any other atherosclerotic treatment. Conclusions based on microarray gene expression profiling were confirmed by immunohistological analyses. Taken together my study identified several previously unrecognized mechanisms, which underlie the atheroprotective activity of captopril.

The second part of my thesis focused on pathomechanisms of heart failure, which is another cardiovascular disease with a high mortality. In frame of our previous studies on pathomechanisms by whole genome microarray gene expression profiling we found a strong up-regulation of the major lipid-synthesizing enzyme, fatty acid synthase (FASN) in experimental models of heart failure induced by major cardiovascular risk factors, i.e. chronic pressure overload and atherosclerosis. This up-regulation of FASN was also a characteristic feature of cardiac biopsies of heart failure patients.

Since the role of FASN in heart failure was not known, the aim of my thesis was to investigate the (patho-) physiological function of FASN up-regulation in the heart. To this end I generated transgenic mice with myocardium-specific expression of FASN under control of the alpha-MHC promoter. Phenotype analysis of the transgenic mice showed that the sole expression of
FASN was sufficient to induce major symptoms of heart failure such as reduced cardiac function, cardiac hypertrophy, and cardiotoxic lipid deposition in cardiomyocytes causing cell death. As the underlying pathomechanism I identified that FASN induced heart failure-specific metabolic changes in vivo and in isolated cardiomyocytes, which could be attributed to activation of the adipogenic transcription factor Pparg (peroxisome proliferator-activated receptor-gamma).

In search for a treatment approach, I analyzed the impact of inhibition of G-protein-coupled receptor kinase-2 (GRK2) because direct inhibition of FASN is not possible due to severe side effects. My data show that inhibition of GRK2 by transgenic expression of a peptide inhibitor of GRK2 (GRKInh) retards the heart failure phenotype induced by FASN. GRK2 inhibition counteracted the activation of Pparg by promoting MAPK-pathway-dependent phosphorylation of Pparg on serine 273. This mechanism was supported by the generation of transgenic mice with expression of the PPARG mutant PPARG-S273A, which does not undergo inactivation by the MAPK pathway. PPARG-S273A mice developed severe signs of heart failure, which were resistant to the beneficial effects of GRK2 inhibition. Thus, the second part of my thesis identified FASN as a previously unrecognized factor in heart failure pathogenesis, which can be targeted by inhibition of GRK2.

Taken together my thesis investigated pathomechanisms of disease in transgenic disease models of atherosclerosis and heart failure. The first part of my study elucidated pathomechanisms of atherosclerosis, which can be targeted by the ACE inhibitor captopril. The second part of my thesis identified FASN as a new player in the lipotoxicity of end-stage heart failure, which can be counteracted by GRK2 inhibition.
2. Zusammenfassung der wissenschaftlichen Ergebnisse

Myokardinfarkt und die sich als Spätfolge daraus entwickelnde Herzinsuffizienz sind die häufigsten Todesursachen weltweit mit nur sehr begrenzten Therapiemöglichkeiten. Das Hauptziel meiner Doktorarbeit bestand in der Identifizierung von neuen Pathomechanismen, aus denen potentielle Zielproteine für therapeutische Ansätze abgeleitet werden könnten.


Meine Studie zeigte, dass Captopril-Behandlung mit 88 ± 7.5 % zu einer stärkeren Reduktion der Größe an atherosklerotischen Plaques in der Aorta führte als Vitamin E, das die Plaquengröße nur um 45.8 ± 11.5 % reduzierte. Während mehr als 30 % der Vitamin-E-regulierten Gen-Sonden übereinstimmend mit Captopril reguliert wurden, zeigten nur 14 % der Captopril-regulierten Gene eine mit Vitamin E übereinstimmende Regulation. Eine Genontologie-Analyse zeigte, dass unter den übereinstimmend regulierten Genen, die antioxidative Kapazität von Captopril und vitamin E dem proliferativen und synthetischen Phänotyp von vaskulären glatten Muskeln entgegenwirkt. Im Gegensatz dazu reduzierte Captopril, aber nicht Vitamin E, die Migration von pro-inflammatorischen und pro-atherogenen Monozyten und T-Lymphozyten in die Aorta. Ausserdem war nur Captopril in der Lage, die Atherosklerose-induzierte Abnahme der Expression von 30 perivaskulären Nerven-spezifischen Genen zu verhindern. Die Schlussfolgerungen aus der Mikroarray-Genexpressionsanalyse wurden durch immunhistologische Analysen bestätigt. Zusammenfassend identifizierte meine Studie einige bislang nicht erkannte Mechanismen, die der atheroprotektiven Aktivität von Captopril zugrundeliegen.

Der zweite Teil meiner Doktorarbeit konzentrierte sich auf Pathomechanismen der Herzinsuffizienz, die eine weitere kardiovaskuläre Erkrankung mit einer hohen Mortalität ist. Im Rahmen unserer vorausgehenden Studien zu Pathomechanismen fanden wir mittels Gesamtgenom-Mikroarraygenexpressionsanalyse eine starke Hochregulation des Enzyms Fettsäure-Synthase (FASN), das hauptsächlich für die Fettsäuresynthese verantwortlich ist, in experimentellen Modellen, in denen Herzinsuffizienz durch die Hauptrisikofaktoren,


Zusammenfassend untersuchte ich im Rahmen meiner Doktorarbeit Pathomechanismen von kardiovaskulären Erkrankungen in transgenen Modellen der Atherosklerose und der Herzinsuffizienz. Der erste Teil meiner Studie klärte Pathomechanismen der Atherosklerose auf, die durch den ACE-Inhibitor Captopril behandelt werden können. Der zweite Teil meiner Arbeit identifizierte FASN als neuen Faktor in der Entstehung toxischer Fettablagerungen bei finaler Herzinsuffizienz, die durch GRK2-Inhibition gemildert werden können.
3. General introduction to common cardiovascular diseases

3.1. Overview of the most common forms of cardiovascular disease

Cardiovascular diseases are the number one cause of death worldwide. Coronary artery disease, myocardial infarction, heart failure and cerebrovascular insult are widespread diseases. Most of the patients have so far been older people in the industrialized countries and Eastern Europe. In parallel with an overall improved quality of life, the incidence and mortality of cardiovascular diseases increase in non-industrialized countries. The overall risk to suffer from a cardiovascular disease as a younger adult increases globally, too. Despite enormous advances in the pharmacological and surgical therapy of cardiovascular diseases since the seventies of the last century, incidence and mortality of cardiovascular diseases are continuously rising in an ageing society. Exploring the pathogenesis of cardiovascular diseases on molecular level can lead to the discovery of new therapeutic principles, making the development of novel drugs possible.

Cardiovascular diseases have many different causes. The spectrum of possible causes spans from genetically inherited diseases over infectious endocarditis to myocardial infarction. One important factor involved in the pathogenesis of the two most frequent cardiovascular diseases, namely myocardial infarction and heart failure, is atherosclerosis. The occlusion of blood vessels by atherosclerotic plaques or their thrombi causes coronary artery disease, myocardial infarction and stroke. On the long-term, coronary artery disease is also one of the main reasons for heart failure, but other important causes exist, too. Diabetic and idiopathic cardiomyopathy are frequent causes of heart failure. The disease mechanisms leading to diabetic and idiopathic cardiomyopathy are not fully understood yet, but metabolic changes play a key role.

In view of the increasing disease burden worldwide my thesis focuses on the study of pathomechanisms to decipher new approaches to the therapy of the two major cardiovascular diseases, i.e. atherosclerosis and heart failure.

3.2 Epidemiology of cardiovascular diseases

3.2.1 Mortality and morbidity of cardiovascular diseases

Over 30% of all deaths worldwide are caused by cardiovascular diseases (CVD) (1). This rate varies between 25% and 30% in high-income countries (2) (3) (4). Many low-income countries have a lower mortality by CVD, about 12%. Eastern European countries and countries with emerging economy show the highest mortality due to cardiovascular diseases (Russian Federation: above 45% in 2011, China: 45% in rural areas, and 41% in urban areas in 2014) (5).

Among different cardiovascular diseases, coronary artery disease (CAD) is the most frequent cardiovascular disease in industrialized countries such as the US, followed by stroke (Figure 3.2.1-1) (6). Meanwhile this notion is valid globally, according to recent data from the WHO (1).

Coronary artery disease (CAD), which is also named coronary heart disease (CHD), is a potentially deadly complication of atherosclerosis and the main cause for myocardial infarction.

According to projections by the WHO and Mathers et al., 33 % of all deaths worldwide will be caused by CVD in 2030 (1) (7). Coronary artery disease (CAD) alone will account for 14% of all deaths, followed by stroke being responsible for 11% of all deaths (8). Taking life quality
into account, CVD were responsible for 12% of all lost disability-adjusted life years (DALY) in 2010 (9). One method to determine the disability-adjusted life-years is to add the years of life living with a disease or disability with the years of life lost by premature death.

Regional differences

The developmental status of a country is linked with changing nutritional and behavioural habits of its population and improved healthcare services. This is reflected by changes in causes of morbidity and mortality concerning CVD and other diseases in general (10). Although the mortality rate due to CVD is higher in the high-income regions of the world like Western Europe, Northern America and Japan, the number of deaths resulting from CVD is much higher in low- and middle-income countries (Figure 3.2.1-2) (11) (12). The largest part of mankind lives in low- and middle-income countries, which are experiencing a rapid increase in atherosclerotic CVD. At the same time the populations of low- and middle-income countries are increasing. In 2001 over 13 million people died from CVD in the low- and middle-income countries, compared to three million people in the high-income countries. The rates of CVD are massively increasing in low- and middle-income countries, with a maximum increase in urban centers. This increase is mainly due to CAD and stroke, which are at least partially caused by the process of atherosclerosis.

Figure 3.2.1-1  Frequency of cardiovascular causes of death (USA, 2011), adapted from Mozaffarian et al., Heart Disease and Stroke Statistics – 2015 Update, Circulation 131, 329-e322 (2015)
2.2 The process of epidemiologic transition

The process in which the pattern of mortality and morbidity changes due to rising living standard is called epidemiologic transition. In general terms, child mortality is reduced and life expectancy increases. Infections and malnutrition are replaced by non-infectious, chronic degenerative diseases, e.g. by cardiovascular diseases or cancer. The term non-communicable diseases (NCD) has been coined for combining these diseases and is widely used in publications by the WHO. Although this concept of “epidemiologic transition” does not apply to all diseases, it in particular reflects the changing epidemiology of cardiovascular diseases in aspiring societies. With increasing prosperity, infectious causes for cardiovascular diseases diminish, while non-infectious causes of CVD - like atherosclerosis - increase (13) (14) (15) (16).

The process of epidemiologic transition can be divided into five consecutive phases, each of it reflects the development of diseases within a population subjected to economic and nutritional changes.

*Figure 3.2.1-2 Frequency of causes of death according to income (adapted from (11)).*
Stage 1 - Pestilence and Famine

In this phase, most deaths occur due to malnutrition and infectious diseases. CVD account for under 10% of all deaths. Many cases of CVD, e.g. rheumatic heart disease, are caused by infections with bacteria or viruses and the resulting inflammation. Cardiac myopathies, caused by infectious agents and/or malnutrition are the most frequent CVD in this phase.

Stage 2 - Receding Pandemics

Deaths caused by infectious diseases and malnutrition are reduced by improved nutrition and better public health efforts by the government. These improvements are accompanied by a massive reduction of infant and child mortality rates. Post-infectious diseases like rheumatic valve diseases are still the dominant CVD, but the cases of CAD, stroke and their risk factor hypertension increase. The proportion of deaths related to CVD varies widely between 10% and 35%, depending on the population studied.

Stage 3 - Degenerative and human-made diseases

Better supply with food leads to increased calorie intake and the consumption of fats is increased. Changing life and working habits result in less physical activity. Consequently, hypertension and atherosclerosis become widespread diseases. Most frequent CVD in this phase are CAD and stroke, the proportion of CVDs caused by infection further decreases. In general, life expectancy increases and non-communicable, chronic diseases are now the main contributors to mortality. Depending on the population under observation, between 35% and 65% of all deaths are caused by CVD.

Stage 4 - Delayed degenerative disease

The treatment options for CVD are improved. Prevention measures are introduced by the government. Less people suffering from CVD die from it. Risk factors for CAD and stroke like hypertension and hyperlipidaemia are controlled by pharmacological measures. The pharmacological and surgical treatment options for patients suffering from CVD are improved. Nevertheless, between 40% and 50% of all deaths are attributed to CVD. In addition to CAD and stroke, congestive heart failure becomes another frequent CVD.

Stage 5 - Inactivity and obesity

Due to changing eating habits and abundance of food, calorie intake is further increased. In parallel, physical activity is even more reduced by lifestyle changes. Many people work in jobs focusing on sedentary activities, e.g. computer work. The results are an increase of overweight and obesity. Diabetes and hypertension are becoming endemic diseases. Atherosclerosis and its risk factors are on the rise, CAD and stroke are the main CVDs. Hypertension and diabetes are risk factors for peripheral vascular disease which becomes one of the major CVD. The cases of congestive heart failure increase. Age-related mortality due to CVD may increase again.

High-income countries with a highly developed medical system are considered to be in the stage of delayed degenerative disease. At this stage, morbidity of CAD remains more or less at a constant level, but age-related mortality due to CAD is reduced by pharmacotherapy, medical interventions and surgical interventions. As a consequence, the proportion of deaths due to CAD in high income countries at this stage varies from 24% to 30%.
3.3. Risk factors of cardiovascular disease

3.3.1 High Blood Pressure

Definition of High Blood Pressure
Hypertension is the elevation of pressure within the vascular system. While the vascular system consists of arteries, capillaries and veins, high blood pressure in its typical definition is a disease of the arterial vascular system (Figure 3.3.1-1). Several classification systems for arterial hypertension exist, which differ in their definition of hypertension and its grades (17) (18) (19) (20).

Prehypertension (18) (21)
Prehypertension is defined as a systolic blood pressure between 120 and 139 mmHg, or a diastolic blood pressure between 80 to 89 mmHg in persons taking no anti-hypertensive medication (Figure 3.3.1-1). The prevalence of prehypertension among persons with no history of CVD or cancer was 36.3% in surveys conducted from 1999 to 2006. Men have a higher prevalence of prehypertension than women. Prehypertension is strongly associated with increased risk factors for CVD and diabetes resulting in an “adverse cardiometabolic risk profile” of affected persons.

This statement is confirmed by the recent “Systolic Blood Pressure Intervention Trial” (SPRINT) which showed that an intensive reduction of blood pressure to a target value of 120 mmHg results in a significantly reduced mortality (21).

Prevalence of High Blood Pressure
High Blood Pressure is a common disease in the US. About 30% of the US adult population have hypertension, amounting to 80 million persons, while 17% of hypertensive individuals were not aware of their condition (22).
The prevalence of high blood pressure increases with age. The prevalence of hypertension in persons 18-39 years of age amounted to 7% in 2011/12 in the US National Health and Nutrition Examination Survey (NHANES). In contrast, persons between 40 and 59 years of age had a prevalence of hypertension of 32%. Persons of 60 years of age or more had an even higher prevalence of 65%. Finally, individuals being 80 years old or older had a prevalence of 77% concerning hypertension (22) (23). Resistant hypertension is found in 12% of hypertensive individuals. They have hypertensive blood pressure values although taking antihypertensive drugs from three different classes or need antihypertensive drugs from at least four different classes to control their blood pressure (24). More older women than older men being at least 65 years of age have hypertension. While 75% of all older women had hypertension, only 65% of older men had hypertension between 2003 and 2006 in the US (25).

The prevalence of high blood pressure in children is naturally lower than in adults. Nevertheless, the prevalence of high blood pressure or borderline high blood pressure in children and adolescents amounted to 11% in 2011/12 (26). High blood pressure is an important comorbidity of diabetes mellitus in childhood and adolescence. The prevalence of high blood pressure among children and adolescents with diabetes mellitus type 2 amounts to 24% and in children and adolescents with diabetes type 1 to 6% (27).

Although widely seen as a disease of adults and the elderly, the AHA recommends measuring blood pressure regularly in certain other groups, e.g. obese children and children suffering from diabetes type 1 or 2 (28). Large racial differences concerning the prevalence of hypertension exist. The age-adjusted prevalence of hypertension was much higher in black men (40.8%) and women (41.5%) than in white men (29.4%) and women (26.5%) in the period from 2011 to 2014 (29).

**High Blood Pressure increases the Mortality from CVD and overall mortality**

According to data from the FHS (Framingham Heart Study) and the NHLBI (National Heart, Lung and Blood Institute) in the US, hypertension reduces the overall life expectancy and “life expectancy free of CVD”. Consequently, hypertension results in more life years with CVD (30).

Prospective follow-up studies conducted between 1975 and 1992 and from 1988 to 2006 showed that hypertensive US adults had a higher mortality than US adults without hypertension (31). The mortality rate for hypertensive individuals was 18.8 per 1000 person-years in the time period from 1975 to 1992 and could be reduced to 14.3 per 1000 person-years between 1994 and 2006. An equal reduction of mortality could be achieved for the normotensive control group, but the mortality of hypertensive individuals was higher (31).

The mortality related to high blood pressure increased by 7% in the period from 2000 to 2013 for persons who were 45 years of age or older. The age-adjusted hypertension-related death rate even went up by 23% between 2000 and 2013 (32). Individuals with pre-hypertensive or hypertensive blood pressure in early adulthood have an increased CVD mortality, CHD mortality and all-cause mortality several decades later (33). This indicates that it might be beneficial to treat hypertensive blood pressure as early as possible (Figure 3.3.1-2).
Risk factors for developing hypertension

Many risk factors have been identified for hypertension. These include non-modifiable risk factors like age, race, family history of hypertension and modifiable risk factors like smoking, physical inactivity, sleep apnea, dietary factors like high sodium and fat intake, overweight and obesity. Psychosocial stressors can also cause hypertension. Analysis of data collected during the Nurses’ Health Study shows that reducing modifiable risk factors including nutrition and lifestyle can prevent the occurrence of hypertension in many women.

3.3.2 Diabetes mellitus

Diabetes is a systemic disease caused by the body’s inability to metabolize glucose sufficiently. The absorption of glucose into the cells is reduced either by insufficient insulin secretion or by decreased effects of insulin secretion on the cells it binds to. Increased and prolonged hyperglycemia after meals results. Overnight fasting glucose levels may also be elevated. Due to increased availability of glucose, more hemoglobin is glycated into HbA1c, which is used as a diagnostic marker. An increased blood glucose concentration and HbA1c level are diagnostic criteria of diabetes (Figure 3.3.2-1). Increased glucose concentrations have deleterious effects on blood vessels, promoting atherosclerosis and the disturbed glucose metabolism can harm cardiomyocytes, promoting heart failure (34).
The longer a person has diabetes, the higher is the person’s risk of morbidity and mortality due to CVD (35). After suffering an acute coronary syndrome, which includes unstable angina pectoris and/or myocardial infarction, patients with diabetes had a higher mortality for 30 days after the event. Even in the first year after the event, patients with diabetes had a significantly increased mortality in comparison to patients without diabetes (36).

Diabetes is also a very important risk factor for developing heart failure, its presence alone qualifies for diagnosis and treatment of stage A heart failure, which is defined by being at high risk to develop heart failure although no symptoms or structural changes of the heart are present (37).

**Prevalence of diabetes mellitus**

The most frequent form of diabetes among adults is diabetes type 2, between 90% and 95% of all cases of diabetes diagnosed in adults belongs into this category (38). The prevalence of diabetes in adults has been steadily increasing in the US population since 1958 (Figure 3.3.2-2). It increased from 6% to 10% between the two periods of 1988-1994 and 2005-2010 (39). The number of persons suffering from diabetes in the US amounted to 21 million adults in 2010 (40). Estimates show that the overall prevalence of diabetes will rise to 12% in 2050. Diabetes has an even higher prevalence among older adults above 65 years of age. The majority of adults in this age group has either diabetes (27%) or prediabetes (50%) (41).
The prevalence of diabetes mellitus type 2 among youths is also increasing. The prevalence of diabetes mellitus type 2 has increased in this population by 30.5% from 2001 to 2009 (42). Most adolescents with diabetes mellitus type 2 are overweight or obese (43). The portion of youths with prediabetes or diabetes mellitus increased from 9% in 1999 to 23% in 2007 (44). Maintaining glycemic control is more difficult in children and youths. Monotherapy with diabetes medication is successful in only 50% of cases (45). The incidence of all types of diabetes in youths was 24.3 per 100,000 person years in the SEARCH study. More than half of the youths diagnosed with diabetes had diabetes type 1 (46). Estimates show that the incidence of diabetes type 1 and diabetes type 2 will further increase (47).

**Diabetes increases the mortality from CAD/CHD**

The mortality rate due to diabetes was 24.0 per 100,000 individuals in 2014 (48). Mortality rates varied between races, the mortality rate of black males and females due to diabetes was higher than the mortality rate for Hispanic males and females. White males and females had the lowest mortality due to diabetes (48).

According to the Framingham Study, the risk to develop CVD is significantly elevated in individuals with diabetes, to similar extent in men (HR 2.4) and women (HR 2.5). After developing CVD, the risk to die was also significantly higher in men (HR 1.7) and women (HR 2.2) with diabetes. Diabetics which were 50 years or older died around 8 years earlier when compared to non-diabetic persons which were 50 years old or older (36).

Cardiovascular disease is the main mortality risk for people with diabetes. Most older adults with diabetes mellitus die of Heart Disease (68%). Death rates due to heart disease are two to four times increased in adults with diabetes (49). A meta-analysis of prospective studies including over 820,000 participants showed that people with diabetes have a higher risk for all-cause mortality (HR 1.25), cancer death (HR 1.25) and vascular death (HR 2.32) (50).
3.3.3. Dyslipidemia and hypercholesterolemia

**Prevalence of high total cholesterol in adults**

Dyslipidemia and hypercholesterolemia are well established risk factors for the development of CVD, hypertension and diabetes. According to the American Heart Association (AHA), the prevalence of elevated total cholesterol (TC) levels measuring 240 mg/dl or more among adults was 12.0% in the time period from 2011 to 2014. Approximately 30.9 million adults have increased TC levels in the US alone. The prevalence of undiagnosed hypercholesterolemia among adults is 6.2%. Mean age-adjusted TC-levels for adults decreased in a linear manner from 206 mg/dl in the period from 1988 to 1994 to 196 mg/dl in the period from 2007 to 2010. The mean serum concentration of TC in adults was 194 mg/dl in men and 198 mg/dl in women in the period from 2007 to 2010. The reduction of cholesterol is primarily attributed to the increased prescription of cholesterol-lowering medications. Common comorbidities of hypercholesterolemia in the US are hypertension (9% of adult individuals) and diabetes mellitus (1.5% of adult individuals) or both (3% of adult individuals) (51).

**Prevalence of high total cholesterol in youths**

7.4% of children and adolescents in the US had high total cholesterol levels of 200 mg/dL or more in measurements conducted in the period between 2011 and 2014 (52).

Evaluation of the NHANE-surveys from 2009-2012 showed that mean TC level among children between 6 and 11 years old was 160.2 mg/dl and the TC level among adolescents aged 12 to 19 years was 158.3 mg/dl (26) (53). Overweight and obese youths have an increased risk for prevalence of abnormal lipid levels. Labtests found at least one abnormal lipid level in 43% of obese youths, in 22% of overweight youths and only in 14% of youths with normal weight. Considering all youths between 12 and 19 years, 21% showed abnormal lipid levels (52).

Analysis of data collected in the two periods from 1988 to 1994 and from 2007 to 2012 found that lipid levels and abnormal lipid levels in children and adolescents aged between 6 to 19 years decreased. Mean TC levels could be reduced from 165 to 160 mg/dl, and the prevalence of elevated TC decreased from 11.8% to 8.1%. The same data was analysed for changes in prevalence of mean LDL-C and elevated LDL-C between the two periods from 1988 to 1994 and 2007 to 2012 in adolescents (53). Total LDL-C decreased from 95 to 90 mg/dl and mean triglycerides dropped from 82 to 73 mg/dl. The proportion of adolescents with elevated LDL-C or triglycerides was significantly reduced. Currently less than one percent of youths are in need of drug therapy to normalize lipid levels (54) (55).

Screening children and adolescents for dyslipidemia is recommended if a risk factor for developing hyperlipidemia or CVD exists. These risk factors are smoking, high blood pressure, overweight or obesity and diabetes. A known family history of dyslipidemia or premature CVD in the family are other risk factors qualifying screening children and adolescents for dyslipidemia (51) (54).

**High total cholesterol increases CAD/CHD mortality**

Numerous studies documented a causal relationship between high total cholesterol levels and mortality from CAD/CHD.
A landmark study on this topic is the “Seven Countries Study”, which strongly supports a direct association between high TC and CAD mortality (56). This study documented for the study cohorts from Northern Europe and United States an exponential relationship between high TC (total cholesterol) levels and an increased risk of CAD mortality (Figure 3.3.3).

**Triglycerides**

The role of high triglycerides in the development of cardiovascular disease is less established. Although many people have high triglycerides, only a minority is treated (57).

### 3.3.4 Smoking

Smoking is considered one of the main risk factors for developing CVD and stroke (58).

#### Prevalence of Smoking

Between 1965 and 2014 the prevalence of smoking among adults could be reduced by half. Smoking has a higher prevalence among males, roughly 19% of all men in the US were smokers in 2014, in contrast to 15% of all women (58) (59). As a consequence of intense prevention and therapy measures the number of cigarette smokers in the US has declined sharply since 1998, smoking prevalence could be reduced from 24.1% in 1998 to 16.9% in 2014. In the US National Survey of Drug Use and Health (NSDUH) conducted in 2013, 5.6% of all participating youths aged between 12 and 17 years indicated “current cigarette use”. About 12% of teenage smokers admitted to consume at least one pack a day. A sharp increase of prevalence is seen in the age group between 18 and 20 years, 27.1% of all adolescent young adults aged between 18 and 20 years of age smoke. The prevalence of smoking cigarettes among young adults has been decreasing since 2003. While 13% of all youths stated to be smokers in 2003, only 5.6% indicated to smoke in the 2013 survey (60).

**Smoking increases the risk of CVD**

Smoking is involved in the pathogenesis of potentially lethal CVD, e.g. CAD/CHD and stroke. Even low-level contact with cigarette smoke results in a significantly increased risk to develop a CVD. The fact whether one smokes or not is more important than the number of cigarettes.
that are smoked. Smoking on its own must be considered an important risk factor for CHD. The effects of smoking in combination with other known risk factors for CHD, like untreated hypertension, diabetes mellitus and hyperlipidaemia, are multiplicative (61). Smoking alone is also an important risk factor for stroke and worsens the effects of other risk factors like elevated systolic blood pressure (62) and oral contraceptives (63) (64). Meta-analyses revealed that the risk of female smokers to develop CHD is higher than for male smokers (65). Female and male smokers have a nearly equal risk to suffer a stroke (66). Their risk is two to four times increased in comparison with non-smokers and persons which stopped smoking over 10 years ago (67) (68). Taken together, tobacco use is considered a main risk factor for disability by the AHA (69).

**Smoking increases the CVD mortality and overall mortality**

Smoking is a risk factor for death. Data from the US document that smoking is a major risk factor for death, surpassed only by dietary risks (69). Smoking and exposure to second-hand smoke are involved in nearly one third of all deaths due to CHD (70). It is well-established that smoking is a risk factor for death caused by the two most common cardiovascular diseases CHD and stroke. New studies show that smoking can also increase the mortality caused by hypertensive Heart Disease (HD) and hypertensive renal disease (71). The life-expectancy of male smokers in the US is 13.2 years lower than for male non-smokers; for female smokers, the life-expectancy is reduced by 14.5 years in comparison to female non-smokers (58). Taken together, smokers have a threefold elevated overall mortality rate in comparison to persons who have never smoked (72).

In 1964, the US Surgeon General published a first report on dangers of smoking. Since then an estimated 8 million premature deaths related to smoking could be prevented by tobacco control measures (73).

**Smoking Cessation**

Smoking cessation is the only way to reduce cardiovascular mortality and morbidity due to smoking and CVDs associated with it. It is recommended to stop smoking at any age. After quitting to smoke, the risk to die from a smoking-related disease decreases continuously (74). Cardiovascular morbidity and mortality are even decreased in patients already suffering from CHD. It is still unclear if the sole reduction of smoked cigarettes per day while continuing to smoke can reduce CVD incidence (61). Giving up smoking between 25 and 34 years of age leads to a 10 year increase in life expectancy in contrast to continuous smokers (72).

**Passive smoking**

Regular inhalation of second-hand smoke increases the risk to develop CHD by at least 25% for non-smokers. Even short-term exposure to second hand smoke can influence coagulation parameters and coronary blood flow (61). The risk to suffer a stroke is increased between 20 to 30% by regular passive smoking (70). The exposure of non-smokers to second-hand smoke was reduced by several measures over the last two decades, including smoke-free laws prohibiting smoking in public places, restaurants and workplaces (75).

**Global burden of smoking**

The global use of tobacco is increasing, although the consumption in the Western hemisphere has been declining. Smoking is currently responsible for 5 million deaths annually. Tobacco use currently causes 5 million deaths a year (76). Tobacco smoke is one of the three leading
risk factors for disease worldwide (77). In order to reduce smoking, the WHO prepared and approved the Framework Convention on Tobacco Control. This convention recommends several measures to achieve this goal, e.g. increasing taxes and establishing educational programs to reduce smoking. It has been in effect since 2005 and 180 states have ratified this WHO convention.

### 3.3.5. Overweight and obesity

Overweight and obesity are major risk factors for CVD, including CAD/CHD, stroke, atrial fibrillation, venous thromboembolism and congestive heart failure (78) (79).

#### Classification of obesity

The unit most frequently used for measuring overweight and obesity is the Body Mass Index (BMI). The BMI is defined as the ratio of body mass in kilograms to the square of body height in meters (kg/m²) and is to a certain extent correlating with the body’s fat content. A synonym used for the BMI is “Quetelet index”. A BMI between 25.0 and 29.9 kg/m² indicates overweight, while a BMI of 30 kg/m² or above equates with obesity (80). For children, BMI values for classification into weight categories change with age and differ between sexes (81).

Another measure to classify obesity is waist circumference (82). Being obese can be defined by waist circumference of 40 inches (102 cm) or above in men, and of 35 inches or more in women. Increased waist circumference is accompanied by an elevated cardiovascular risk. Especially in the BMI range between 25 and 34.9 kg/m², an increased waist circumference indicates an increased risk for CVD (80).

#### Prevalence of obesity

Assessment of the NHANES (National Health and Nutrition Examination Survey) data collected from 2009 to 2012 revealed that 69% of all US adults were overweight or obese. Obesity was prevalent in 35% of all US adults. There are pronounced regional differences in the prevalence of obesity. According to self-reported data, only 21.3% of inhabitants of Colorado were obese, but 35.1% of the population in West Virginia. Self-reported data are often inaccurate concerning BMI and obesity because these two values are often underestimated.

In addition, analysis of the data collected by the NHANES in 2011/2012, which was conducted by the National Center for Health Statistics, showed that 31.8% of all children and adolescents between 2 and 19 years old were overweight or obese. Notably, the prevalence of overweight and obesity was 22.8% in the group of young children aged 2 to 5 years but increased to 34.2% in the group of children aged 6 to 11 years and stayed at 34.5% in the group of youths aged 12 to 19 years. Evaluation of the same data showed that 16.9% of children and adolescents were not only overweight, but obese. Prevalence of overweight and obesity was the same in boys and girls (83).

Analysis of NHANES data shows that the prevalence of obesity in adults has not changed from the period of 2003-2004 to the period of 2011-2012 (81) (83). However, prevalence of obesity increased from 31.5% to 38.1% in the subgroup of women aged 60 years or older. The prevalence of overweight and obesity in infants, children and youths has been relatively stable since 2003 (83) (84).
Obesity - a risk factor for CAD/CHD

In a meta-analysis including 58 cohorts and 221,934 participants’ parameters for measuring adiposity, namely BMI, waist circumference and waist-to-hip ratio were strongly associated with risk factors for cardiovascular diseases like diabetes mellitus, higher systolic blood pressure, increased total cholesterol and lower HDL-cholesterol. Adiposity measures were strongly associated with CAD/CHD, ischemic stroke and other CVD in the same studies. Furthermore, obesity is a risk factor for subclinical atherosclerosis (85). The Multi Ethnic Study of Atherosclerosis (MESA) found increased measurements for coronary artery calcification (CAC) and carotid intima-media thickness in obese participants. These effects where independent from other CVD risk factors (86). Obesity is linked with an increased incidence of sleep-disordered breathing, which is itself a risk factor for CVD (87). Overweight and obese individuals have an increased risk to suffer ischemic stroke. The risk for suffering hemorrhagic stroke is increased in obese individuals. The higher the BMI, the higher the risk for developing stroke. Age, lifestyle and other lifestyles had no major influence on this result (88).

The risk to develop severe obesity as adults is significantly increased in obese adolescents when compared with normal weight adolescents, i.e. their probability to have severe obesity in their adulthood is 16 times higher. Thus, many adolescents with severe obesity continue being obese as adults (89). The risk of overweight or obese children and youths to develop cardiovascular risk factors like hypertension, hyperlipidaemia and diabetes mellitus in the future is also increased (90). The risk for overweight or obese children and youths to suffer from other diseases or conditions related to CVD like asthma and sleep apnoea as an adult are higher, too. Cohort studies which examined the development of BMI came to the conclusion that overweight or obese children, who became obese or continued to be obese in adulthood had an increased risk of developing cardiovascular risk factors. In contrast, overweight or obese children who were able to reduce their weight to a normal level by reaching adulthood had no increased risk of developing cardiovascular risk factors (91).

Adults with overweight and obesity have a higher prevalence of diabetes mellitus, hypertension and dyslipidemia. The higher the BMI, the higher the risk of diabetes mellitus type 2 (92). Cardiovascular risks may increase with the severity of obesity. Postmenopausal women suffering from grade III obesity with a BMI of 40kg/m² or more had a massively increased risk to develop chronic heart failure (CHF) and non-fatal CHD, although the CHD risk was influenced by additional CVD risk factors (93).

Obesity increases the risk of all-cause mortality

Analysis of National Health Interview Surveys (NHIS) showed that obese persons have an elevated risk to die, their all-cause mortality is increased. (94) (95). The hazard ratio for all-cause mortality in self-reported obese individuals was 1.41 and for extremely obese individuals 2.46. A large systematic review incorporating meta-analysis of large studies showed that all-cause mortality was increased for obese individuals in comparison to individuals with normal BMI. The lowest mortality could be found at a BMI between 22.5 and 25 kg/m² in men and women of all age groups (96). Obesity is associated with a higher mortality due to CVD, some types of cancer, diabetes mellitus and kidney disease. Obesity accounted for 13% of CVD deaths in 2004 (97). Obesity combined with other metabolic dysfunctions is more harmful than obesity alone. Obese individuals, who are “metabolically unhealthy” have an elevated all-cause mortality in comparison to “metabolically healthy” obese individuals (HR 1.72) (98). Patients who suffered a STEMI and were highly obese (>40kg/m²) had a higher in-hospital mortality rate than STEMI patients with low-grade obesity.
Some studies suggest that waist circumference or waist-to-hip ratio are more accurate markers for evaluating the risk of mortality due to CVD than BMI alone. While the risk of death per SD increase was 1.12 for the BMI, the risk of death per SD increase was 1.19 for waist circumference and 1.23 for the hip-to-waist ratio (66). Waist circumference is in some way associated with mortality. A gain of 5 cm waist circumference is associated with increased all-cause mortality, independent from the BMI range (99). In postmenopausal women with severe obesity (BMI equals 40 kg/m² or more) increasing waist circumference above 115 cm was associated with climbing mortality and incidence of CHD and CHF (93). In the Atherosclerosis Risk in Communities (ARIC) Study higher BMI values and increased waist circumference were associated with the risk of sudden death (100).

**Global outlook**

The prevalence of overweight or obesity had been increasing worldwide between 1980 and 2013. The share of overweight and obesity increased from 28.8% in 1980 to 36.9% for men and from 29.8% to 38% for women in 2013 (101). The mean BMI of men has increased by 0.4 kg/m² per decade in the period between 1980 and 2008, while the mean BMI of women even increased by 0.5 kg/m² per decade in the same time period (102). The number of deaths attributable to elevated BMI increased by the factor 1.7 between 1990 and 2010, to about 3,400,000 deaths registered in the US alone. Approximately 1.46 billion people were overweight or obese in 2008. This number includes 205 million obese men and 297 million obese women (103).

**3.3.6. Physical inactivity**

Physical inactivity is also a major risk factor for CVD and stroke (104).

**Definition of physical activity**

There are four dimensions of physical activity (type, frequency, duration, intensity) and four common domains (occupational, domestic, transportation, leisure time). It is important to generate data on all dimensions and domains of physical activity (105). There are two methods to assess physical activity: subjective methods that use questionnaires or diaries, and objective methods that use wearable monitors (e.g. pedometers). Often marked discordance between reported and measured physical activity exists, with respondents often overstating their physical activity, especially the intensity (106) (107) (108). Chronic physical inactivity contributes to a poor level of cardiorespiratory (or aerobic) fitness, which is a stronger predictor of adverse cardiometabolic and cardiovascular outcomes than additional risk factors. Both physical activity and cardiorespiratory fitness are inversely related to the risk of CVD and other clinical outcomes, they are in part distinct measures in the assessment of CVD risk (109).

**Prevalence of physical inactivity**

Physical activity (PA) among adults is decreasing. The percentage of male adults being physically active for over 12 times a month shrunken from 57% to 43.3% between the two periods of 1988-1994 and 2001-2006 (110). Women showed the same tendency, but with lesser extent. In 2014, 30.2% of adults reported not to engage in any “leisure time PA”, which is defined as physical activity for at least 10 minutes (111). Physical inactivity increased with age, over 50% of persons older than 75 years were physically inactive (111). Only 21.4% of adults were able to reach the goals set by the federal guidelines for physical activity and strengthening activity in 2014 (111). The US federal guidelines for physical activity recommend at least 150 min of moderate physical activity or 75 min of vigorous physical activity each week. These aims were only reached by 50% of adults in the US in 2014 (105) (111).
In a survey conducted in 2013, 15.2% of adolescents in the US indicated to have been physically inactive for the last 7 days. The percentage of inactive girls (19.2%) is higher than those of boys (11.2%) (112). Only 27.1% of high school students where physically active for more than one hour a day on 7 days of the week, and therefore met activity recommendations by the AHA and the 2008 federal guidelines (112). Watching TV, playing computer games and using the internet are sedentary activities connected with physical inactivity. Computer use for over three hours a day by youths increased between 2003 and 2013 by 19%. In 2013, 41.3% of adolescents in the US used the computer for leisure activities for over three hours a day on school days, in comparison to 22.1% of youths in 2003. Watching TV for over three hours a day is common practice for 32.5% of adolescents (112). As a consequence of less physical activity, the number of youths with “adequate levels of cardiorespiratory fitness” decreased from 52.4% in 1999 to 42.2% in 2012 (113).

Association of physical inactivity with CVD and metabolic risk factors

The risk for adults to develop the metabolic syndrome can be decreased by regular physical activity of moderate intensity amounting to 120-150 minutes per week. Physical activity improves insulin sensitivity and reduces dyslipidemia, hypertension and overweight, which are risk factors for developing the metabolic syndrome (114)(115)(116). Physical activity leading to weight loss improves measurements for diastolic blood pressure, triglycerides and fasting glucose (117). Persons, who complied with the federal recommendation to be physically active for at least 150 minutes per week, had a 44% risk-reduction to develop diabetes mellitus after a follow-up of 3.2 years (118).

Youths which are regularly physically active have less body fat and are in fewer cases obese (119). The more physically active children between 4-18 years are, the more ideal are critical metabolic factors like waist circumference, systolic blood pressure, fasting triglycerides and high density lipoprotein. Increasing physical activity and decreasing sedentary activities of children and youths resulted in improved “metabolic risk factor profiles” (120).

Physical inactivity increases morbidity from CVD

Physical inactivity is one of the main factors for suffering myocardial infarction worldwide (121). Sedentary activities are associated with an increased morbidity for diabetes and increased overall mortality. Stroke is less likely to occur in persons with regular physical activity (122). Several longitudinal studies show an inverse association of physical activity with CAD and stroke, which is dependent on intensity and duration (122) (123). A certain “dose-effect” of physical activity could be examined (124). “Sitting for >10h” a day and low physical activity was “associated with increased CVD risk” (125). The researchers came to the conclusion, that long-time sitting and low physical activity increase the risk for CVD (125). Obese persons with first symptoms of the metabolic syndrome profit from physical activity. Physically active obese persons with metabolic syndrome had a 50% lower risk to develop CAD when compared to obese persons with metabolic syndrome leading a sedentary lifestyle in the EPIC-Norfolk study (126).

Physical inactivity is associated with a higher mortality

Estimates show that 5% of all deaths associated with CAD could had been prevented by a 2.3% decline of physical inactivity between 1980 and 2000 in the US alone (127). In the Cooper Center Longitudinal study, which included over 16000 participants, a low cardiorespiratory fitness led to an increased risk of mortality due to CVD. The mean follow-up time was 28 years
In the Southern Community cohort study over 12 h of sedentary activities a day where associated with a 20% to 25% higher risk of overall mortality. While PA was inversely correlated with mortality risk, sedentary time was associated with increased mortality (129). A large study with 55,000 adult participants showed that regular, moderate physical activity, e.g. a 10-minute run with low speed, can reduce the overall mortality and the mortality due to CVD. Even moderate physical activity is beneficial (130).

**Global outlook - physical inactivity is increasing**

Globally, physical inactivity is rising. Meanwhile, the worldwide prevalence of physical inactivity (35%) has surpassed the worldwide prevalence of smoking (26%) (131).

### 3.3.7 Nutrition

**Effects of diet on cardiovascular risk factors including diabetes mellitus type 2**

Modified DASH-type diets (Dietary Approaches to Stop Hypertension) with either increased unsaturated fat or protein content had beneficial effects on CVD risk factors comparable to the low-fat DISH-diet™. All DASH-diets are largely based on unprocessed foods like fruits, vegetables, whole grains, fish and include potassium supplementation while sodium intake is low. This type of diet has beneficial effects on several cardiovascular risk factors. It decreased the systolic blood pressure (SBP) by 8-10 mmHg, the diastolic blood pressure (DBP) by 4-5 mmHg and the LDL-C by 12 to 14 mg/dl. Triglyceride levels could be reduced by DASH-diets with higher content of proteins (up to 16 mg/dl reduction) or unsaturated fats (up to 9 mg/dl reduction) (132). The variation of the fat content alone (27-37%) had no significant effect on blood pressure or LDL concentration. Increased intake of trans-fats instead of saturated fat, monounsaturated fat or polyunsaturated fat can lead to an increased TC to HDL ratio and elevated serum levels of apolipoprotein B (133). Regular intake of eicosapentaenoic acid and docosahexaenoic acid for a duration of 212 weeks reduced the systolic blood pressure by 2.1 mmHg (134). According to several randomized trials, nut consumption has positive effects on blood lipid levels (135) (136). The daily consumption of 67 g of nuts resulted in decreased levels of total cholesterol, LDL-C and a reduced TC-to-HDL ratio. Serum triglyceride concentration could be reduced in individuals with high triglycerides. The type of nuts consumed was not significant for the effect (136). Mediterranean dietary patterns incorporating virgin olive oil or mixed nuts showed to have effects on different risk factors for CVD. First, blood pressure could be reduced. The SBP could be reduced by 5.9 mm Hg in the olive oil group, and by 7.1 mmHg in the nuts-consuming group. Secondly, positive effects on lipid metabolism could be detected. The TC-HDL ratio decreased by 0.38 and 1.6 mg/dl, respectively. The levels of HDL-C rose by 2.9 mg/dl in the olive oil-group and by 1.6 mg/dl in the nuts-consuming-group. Third, glucose metabolism was improved, plasma glucose could be reduced by 7.0 mg/dl and 5.4 mg/dl (137).

**Increased carbohydrate intake increases the risk of diabetes mellitus type 2**

Increased consumption of refined complex carbohydrates and sugars was found to be associated with a higher risk of developing diabetes mellitus type 2 by 24 prospective cohort studies. The intake of carbohydrates was measured by determining the glycemic loaf of the foods consumed (138).

**Effects of fats and carbohydrates on cardiovascular outcome**

Several randomized clinical trials came to the conclusion that reduction of total fat consumption has only little influence on the risk of CVD (139). In the Women Health Institute
clinical trial, which included over 480000 women as participants, reducing fat consumption had no influence on the incidence of CHD, stroke or total CVD (140). The incidence of CHD, stroke and total CVD also was not related to the overall uptake of saturated fats in three independent meta-analyses of multiple prospective cohort studies (141) (142) (143).

Nevertheless, replacement of saturated fats with polyunsaturated fats was associated with a lower risk to develop CHD in an analysis of 11 prospective cohort studies. A 5% energy exchange of saturated fats with polyunsaturated fats led to a risk reduction of 13% to develop CHD (144). In contrast, the substitution of saturated fats with carbohydrates showed to be disadvantageous. A 5% energy exchange of saturated fats with carbohydrates increased the risk to develop CHD by 7% (144). The replacement of saturated fats or carbohydrates with polyunsaturated fats reduced the risk of developing CHD in the meta-analysis of cohort-studies (145). The consumption of trans-fats increases the risk to develop CHD massively. Every 2% of calories taken up in the form trans-fats increases the risk to develop CHD by 23% (146).

The consumption of complex carbohydrates

Consumption of refined complex carbohydrates, starches and sugars was associated with a higher risk for CHD and diabetes mellitus. Risk to develop CHD was increased up to 36% in the high carbohydrate intake group, the risk to develop diabetes was also up to 40% increased (147) (148).

Foods and beverages

By eating one serving of fruits or vegetables daily, the risk to develop CHD could be decreased by 4%, and the risk to develop stroke could be decreased by 5% (149) (150). An increased consumption of 2.5 servings of whole grain showed to reduce the risk for CVD events, like CHD, stroke and fatal CVD (151). Eating fish regularly decreased the risk to die from CHD (152). The daily intake of fish containing 250 mg of long-chain omega-3 fatty acids lowered the risk of mortality due to CHD by 35%. Consumption of unprocessed red meat had no significant effect on the incidence of CHD. On the contrary, the intake of 50 g of processed meat like sausages or bacon on a daily basis, increased the incidence of CHD (153). Notably, the risk to develop diabetes mellitus was increased seven-fold by the intake of 50 g/d of processed meat (154). In contrast, regular consumption of nuts proved to be protective against the incidence of non-fatal and fatal CHD in several studies, while nut consumption did not lower the incidence of stroke. The intake of 100 g of legumes four times a week also reduced the incidence of CHD (155). A large study including over 88.000 women showed that drinking sugar-sweetened beverages daily is associated with higher risk for CHD (134).

Sodium and potassium

Sodium can increase blood pressure dose-dependently. The effects of sodium-intake effects are more pronounced in the elderly and hypertensive persons (156). Additionally, sodium causes damage to the vasculature and kidney, involving other mechanisms than increased blood pressure (157). Diets low in sodium content, e.g. the DASH-type diets, can decrease systolic blood pressure by 7.1 mmHg in normotensive adults. The effect on adults with hypertension was even stronger, their systolic blood pressure could be reduced by 11.5 mmHg.

Potassium lowers blood pressure, especially in hypertensive persons. Diets rich in potassium reduce the risk of CVD, particularly stroke (158) (159). Sodium intake higher than 4000 mg per day is associated with increased CVD events, especially stroke (160) (161). Feeding trials showed that a reduction of sodium intake results in a decreased blood pressure until the uptake
of 1500 mg/d is reached (162). Depending on the prospective observational studies analysed, the value until which reduction of sodium intake results in a reduced risk of CVD varies between 1787 to 2391 mg/d (160) (161).

One large sodium study noticed that a sodium intake of less than 2300 mg/d reduced the risk of CVD by 32% in comparison to sodium intake of 3600 to 4800 mg/d (163).

Nevertheless, data on sodium intake appear conflicting. Several studies showed an increased risk for CVD for low sodium intakes and high sodium intakes, with maximal risk reduction in between. This would result in a J-shaped relationship of sodium intake with CVD risk (164) (165) (166) (167). New studies evaluate the effect of sodium intake on mortality due to CVD in hypertensive and non-hypertensive individuals. The results suggest that only patients with increased blood pressure and consuming more than 6 g sodium per day profit from a reduction of salt intake. Adhering to a low-salt diet with less than 3000 mg intake per day increased CVD mortality for hypertensive and normotensive participants of the study. To which extent sodium intake should be tolerated under which circumstances is still matter of discussion.

**Dietary patterns**

Individuals keeping a “Mediterranean” dietary pattern rich in vegetables, legumes, nuts fruits, whole grains, fish and unsaturated fat, but containing only little meat, had a lower incidence for CHD and stroke. Their mortality due to CVD was reduced by 22% (168). Adherence to a DASH-type diet showed similar effects (169). Shifting to a “Mediterranean-style diet” was beneficial for patients with existing CVD risk factors and resulted in a risk reduction of 30% for stroke, myocardial infarction and death due to CVD (170).

**Impact of diet and nutrition on cardiovascular risk**

Unfavourable nutrition was the primary cause of mortality and was responsible for most lost DALYs in 2010, it even surpassed smoking as cause of mortality in the US and worldwide. In 2010, suboptimal nutrition was involved in the death of 678.000 individuals in the US. High sodium intake is estimated to have been responsible for 102.000 annual deaths in 2005 alone (69) (171).

4.1. Introduction into atherosclerosis and Coronary Artery Disease (CAD)

4.1.1 Pathomechanisms of atherosclerosis and Coronary Artery Disease

My work is focused on the two most frequent cardiovascular diseases, which are coronary artery disease and heart failure. The diagnosis, treatment and general pathophysiological background of these two diseases will be presented in the following two chapters. Coronary artery disease (CAD) will be discussed first, as its pathology resembles the disease mechanisms of other atherosclerotic diseases, e.g. stroke. Additionally, severe coronary artery disease often precedes and causes the development of heart failure later in life.

Atherosclerosis is a pathological process affecting the walls of the arteries. The deposition of lipids leads to local endothelial dysfunction, invasion by leukocytes and to proliferation of local tissue cells. The vessel wall becomes hypertrophic and rigid. An atherosclerotic plaque can emerge, which can further reduce the vessel's lumen. Blood flow through the artery is decreased, oxygen supply for dependent organs is reduced. If the atherosclerotic plaque is unstable, it can get injured, leading to further complications.

4.1.1.1 Stages of atherosclerosis

The process of atherosclerosis is sequential and can be divided into stages. Most frequently applied in science is the classification system published by the American Heart Association in 1995 (172). It includes categories and descriptive terms (Figure 4.1.1.1-1). Other classification systems exist, depending on the type of pathological changes being in the focus of interest. In addition to the classification scheme, Figure 4.1.1.1-2 shows the different lesion types, and a proposed sequence of their development during the pathogenesis of atherosclerosis and atherosclerotic plaque formation (173).

4.1.1.2 Keyplayers of atherosclerosis

Dysfunctional Endothelium

The endothelium consists of all endothelial cells which are lining arteries, veins and capillaries of the body. Endothelial cells have many physiologic functions, including barrier functions, regulatory functions concerning blood coagulation, and immunologic functions (174). Intact healthy endothelial cells are antiatherogenic and anti thrombotic (175) (176) (177). If the physiological conditions of endothelial cells are disturbed their properties change (178). Risk factors for atherosclerosis like hypertension, hyperlipidemia and diabetes are triggers of endothelial dysfunction (179). Dysfunctional endothelial cells change their phenotype to a "proinflammatory and prothrombotic state", promoting adhesion of leukocytes to them. (180) (181). Dysfunctional endothelial cells themselves have a reduced synthesis of nitric oxide (NO), formerly known as endothelium-derived relaxing factor (182) (183).
At the same time, the proatherogenic and vasoconstrictive peptide endothelin-1 is expressed at increased levels by dysfunctional endothelial cells (184) (185). The endothelium’s adhesion abilities are increased and its ability to regulate vessel diameter is compromised. Dysfunctional endothelial cells increase their expression of adhesion molecules like P-selectins, E-selectins, VCAM-1, ICAM-1 and PECAM (CD31), which facilitate rolling and attachment of leukocytes to them (108) (186) (187) (188) (189). Additionally, synthesis and secretion of cytokines attracting monocytes, e.g. "monocyte chemoattractant protein-1" (MCP-1), is increased (190) (191). At the same time, dysfunctional endothelial cells produce more PAI-1 (plasminogen activator inhibitor-1), promoting local blood coagulation (192). Activated endothelial cells in early atherosclerotic lesions show "increased numbers of micro-vesicles in their cytoplasm", probably indicating endocytosis of LDL particles, possibly a result or sign of endothelial dysfunction. The permeability of the dysfunctional endothelium for LDL particles is increased. LDL particles leave the flowing blood, pass the endothelium and deposit in the sub-endothelial intima (187) (188).
LDL deposition and monocyte migration

LDL particles, which are rich in cholesterol, can penetrate dysfunctional endothelium. They deposit in the artery's intima, the tissue layer bordering the endothelium (193). As a reaction to LDL deposition in their vicinity, endothelial cells express adhesion molecules at an increased level. Monocytes are attracted to the site of LDL deposition and migrate into the intima from the luminal side after attaching to adhesion molecules expressed by the endothelial cells (194). By passing from the blood into the intima, they transform into macrophages and head for the LDL molecules. They are guided by chemotactic molecules, which are set free by other cells irritated by the deposited LDL particles (195) (196). After having arrived in the intima and sub-intima the macrophages begin to take up LDL particles by phagocytosis (197). LDL particles have a high affinity for the macrophage scavenger receptors A and B, bind to them and are taken up (198). The macrophage incorporates more and more LDL particles and is not able to digest them. As a consequence, the macrophage degenerates into a rather immobile, lipid-loaded "foam cell". After their apoptosis, inflammatory mediators are released which promote inflammation (199) (200) (201).

Vascular smooth muscle cells

Only few vascular smooth muscle cells can be found in the intact arterial intima. Upon deposition of LDL in the intima, local vascular smooth muscle cells (VSMCs) begin to proliferate, and many more migrate from the media into the intima (202) (203) (204). Having arrived there, they proliferate, express adhesion molecules and take up LDL by phagocytosis (205) (206) (207) (208). Normal smooth muscle cells of the media contain many myofilaments and are of "contractile phenotype". After migrating into the intima and being exposed to LDL,
they change their phenotype and become highly active in synthesizing constituents of connective tissue, e.g. collagen, elastin and proteoglycans (209) (210). This type of smooth muscle cells is responsible for producing the fibrous matrix of atherosclerotic plaques and is hence termed "synthetic phenotype". Smooth muscle cells are the main source of "collagen-rich fibrous tissue" in plaques, which gradually replaces the "loose fibrocellular tissue" found in healthy intima.

### 4.1.1.3 Role of the innate immune system in atherosclerosis

#### 4.1.1.3.1 Monocytes

Monocytes exert a major role in the pathogenesis of atherosclerosis. Monocytes circulating in the blood are attracted to the endothelium which has been activated by LDL deposition in several ways (Figure 4.1.1.3.1).

![Figure 4.1.1.3.1 Scheme of monocyte recruitment and accumulation in atherosclerotic plaques (from Moore et al., Nat. Rev. Immunol. 13, 709-721 (2013).](image)

Inactive monocytes normally pass an arterial blood vessel in its "central axis of blood flow". Lipoprotein(a) in atherosclerotic plaques can lure leukocytes into the intima (211). Chemoattractants released from the intima inflamed by LDL deposition leak into the artery and stimulate the monocytes to actively move into the "peripheral marginal bloodstream" by directed migration. In accordance with this observation, Smith and Huo showed that CXCL-1 can arrest monocytes from a flowing current in a flow cell (212). Endothelial cells overexpress cell adhesion molecules after activation, increasing the chance to bind monocytes. This can either happen by shear stress, LDL deposition in the intima or both (187) (213) (214) (215). One principal chemoattractant for monocyte adhesion to the endothelium and their transendothelial migration is MCP-1 (216).
The adherence of monocytes to the endothelium is a process consisting of attachment, rolling and firm adherence, which is followed by migration of the monocyte through the endothelial layer into the arterial intima (217). First contact ("tethering") between a monocyte and the endothelium is achieved by selectins, which are expressed by both cell types. The monocytes carry L-selectin on their membrane, while activated endothelial cells express E-selectin and P-selectin on their luminal membrane (108) (218). The main ligands of these selectins are "fucosylated and sialylated carbohydrates", which are present on monocytes and activated endothelial cells, allowing attachment of the monocyte to the endothelium. After having established contact with the endothelium, the monocyte is activated and moves along the endothelial layer by rotating around itself. This process is termed "rolling" and increases contact surface between the membranes, leading to further activation of the monocyte. Nevertheless, the binding between monocyte and endothelium is still weak. Responsible for the firm adhesion between monocyte and endothelium are a second class of cell adhesion molecules, the integrins, and their ligands. Integrins of beta-1 and beta-2 class are expressed by monocytes. Their affinity towards their ligands is highly increased upon the cell's activation. Endothelial cells carry ICAM-1 and VCAM-1 on their surface, which are ligands for integrins (186). At the same time, endothelial cells in inflammatory state overexpress integrins themselves. The interaction between the integrins and their ligands ICAM-1 and VCAM-1 leads to firm adhesion between monocyte and endothelium (219) (220). Adhesion of the macrophages to the endothelium is also enhanced by Fc-receptors and complement receptors, which are expressed at increased numbers by activated monocytes (221) (222). During the process of adhesion, monocytes develop small protruding pedicles, in reaction to stimulation. The pedicles increase the area of adhesion and support sensing molecular stimulants from the endothelial cells and the intima beneath them. The process of adhesion is followed by the monocyte's migration through the layer of endothelial cells into the intima. The process of migration is facilitated by platelet adhesion molecule-1 (PECAM-1) and junctional adhesion molecule-A (JAM-A), which both belong to the group of immunoglobulin proteins (108) (189) (223) (224). These two adhesion molecules are expressed by activated monocytes and by endothelial cells. The density of PECAM-1 and JAM-A is increased at cell contacts between endothelial cells. During migration, the PECAM-1 and JAM-A molecules on monocytes and endothelial cells interact homotypically and allow "transendothelial diapedesis" (189) (223). The monocytes are able to penetrate the endothelial layer by squeezing themselves through the space between two endothelial cells. Inflamed endothelial cells show a "redistribution" of PECAM-1 and JAM-A to their apical surface, thereby enhancing monocyte migration (223). Other adhesion proteins like junctional adhesion molecule-C (JAM-C) and lymphocyte function-associated antigen-1 (LFA-1) are also involved in monocyte migration (225) (226). JAM-A is expressed by endothelial cells and undergoes homotypic binding with JAM-A of neighbouring endothelial cells thereby tight junctions between endothelial cells are stabilized by homotypic binding between JAM-A adhesion molecules. Monocytes which carry LFA-1, an alpha/beta-integrin, can interrupt these homotypic binding because LFA-1 has a stronger affinity for JAM-A, leading to breaking of the bonds between JAM-A adhesion molecules, resulting in weakening of the tight junctions (227). JAM-C is also found on endothelial cells and can bind to Mac-1, which is a beta-2 integrin on the membrane of macrophages and neutrophil granulocytes. The expression of JAM-C on cultured human arterial smooth muscle cells (HASMC) and endothelial cells is up-regulated by ox-LDL in vitro in studies conducted by Keiper et al., indicating it is involved in the process of atherosclerosis (225).

**Monocyte differentiation within the intima**

Monocytes, which have migrated into the arterial intima under the influence of MCP-1 differentiate into macrophages by being subjected to M-CSF and other cytokines or chemokines in the intima (228) (229) (230). The differentiation results in changed appearance
and function. Macrophages are larger than monocytes, their cytoplasm is expanded and contains increased numbers of vacuoles, vesicles and lysosomes. The macrophages encounter lipoprotein particles like LDL and take them up by phagocytosis, leading to further activation and differentiation of the macrophage. The macrophage’s differentiation is also influenced by cytokines released by other immune cells, VSMCs and endothelial cells (231). Cholesterol is taken up by scavenger receptors, CD36 and SR-A, and is transformed into cholesterol ester by the enzyme “acyl-coenzyme A:cholesterol acyltransferase-1” (ACAT1) upon its arrival at the endoplasmic reticulum (232) (233) (234) (235). Oxidized LDL-cholesterol (oxLDL) exerts stronger stimulating effects on monocytes and macrophages, and is taken up by the lectin-like oxLDL receptor-1 (LOX-1). The cholesterol esters are then stored in the shape of cytoplasmic lipid droplets. The enzyme catalyzing the reverse reaction, i.e. the hydrolysis of cholesterol esters into free cholesterol is “neutral cholesteryl ester hydrolase” (nCEH) (236) (237) (238). After hydrolysis of the cholesterol esters, the free cholesterol is ready for transporter-mediated release from the cell. Due to their inability to degrade cholesterol sufficiently and limited activity of nCEH, macrophages accumulate lipid particles in their cytoplasm and increase further in size. The appearance of these macrophages under the microscope is of undefined shape, the cells are filled with phagosomes containing lipids. These rather inactive and apoptosis-prone cells release proteases, elastases and modified lipids, which accelerate atherosclerosis and the growth of atherosclerotic lesions (239). As a consequence of their appearance, these macrophages are called "foam cells".

4.1.1.3.2 Complement

The complement system is part of the innate immune system and can target antigens either directly by forming heteropolymers composed of different complement proteins which attach to it, or indirectly by activating leukocytes of the innate immune system (240). The complement system includes complement factors and complement receptors. Three different cascades of enzymatic complement activation exist, but all of them result in the generation of complement factor C3, thereby promoting the generation of the terminal complement complexes (“TCC”), the most well-known being the membrane attack complex (“MAC”). These complement complexes can insert into the membranes of targeted cells and damage them (241) (242) (243).

Contact with bound toxins, immunoglobulins, membrane structures or other activated complement components can lead to activation of complement factors, which bind to the toxin or antigen and stimulate leukocytes by binding to complement receptors, resulting in inflammation, increased phagocytic activity of macrophages and possibly apoptosis of targeted cells (240).

Complement and its regulators in atherosclerosis

The walls of atherosclerotic arteries contain a wide variety of proteins involved in complement activation and complement regulation (244). Several investigations showed that complement factors, including their regulators and receptors can be found in atherosclerotic plaques at increased levels (245) (246). The gene transcription of complement factors is also elevated in atherosclerotic plaques, more mRNAs coding for components of the complement system could be detected in atherosclerotic lesions and atherosclerotic artery walls than in unaffected arteries (247) (248) (249). Complement factors can be synthesized locally in the atherosclerotic plaque and can be retained from the circulating blood and lymphatic fluids (250).

Some complement factors are expressed by many types of cells involved in the pathogenesis of atherosclerosis. The complement factor C1q is expressed by dendritic cells, macrophages, foam cells and neovascular endothelial cells (251). The TCC C5b-9 is present in atherosclerotic arterial walls and can be detected with antibodies (252) (253). Regulators like vitronectin and
Complement factor H, which can limit effects of complement activation, bind to matrix proteins of the connective tissue within the intima (254). If vitronectin and factor H co-localize, like in the superficial intima of early atherosclerotic plaques, the formation of C5b-9 may be reduced (255).

Complement activation through the classical pathway in atherosclerosis

The predominant pathway of complement activation in atherosclerosis is the classical pathway. The C-reactive protein (CRP) and antigen-antibody complexes are efficient activators of this pathway. In atherosclerotic plaques, CRP bound to modified LDL and antibodies bound to oxLDL or heat shock proteins are abundant, facilitating activation of the complement system (256) (257). Large amounts of CRP deposit in the “atherosclerotic arterial intima” and high levels of mRNA coding for CRP can be detected here (258) (259). There, CRP co-localizes with the terminal complement complex C5b-9. As mentioned before, the expression of CRP is elevated in atherosclerotic plaques, mRNA levels coding for CRP are increased 10-fold in fibrous plaques. CRP then binds to oxidized LDL, enzymatically modified LDL and apoptotic cells, resulting in activation of the complement system and the generation of C5b-9 complexes which are promoting atherosclerosis (257) (260). Autoantibodies directed against and bound to oxidized LDL or heat-shock protein 60/65 can also activate the complement system through the classical pathway, which may play a role in atherosclerosis (261). This pathological mechanism is frequently found in patients with SLE, which are prone for the development of atherosclerosis and other cardiovascular diseases (262) (263).

The alternative pathway

The presence of apoptotic cells in fibrous plaques and necrotic cores and their co-localization with C5b-9 complexes suggests that complement is also activated by the alternative pathway in atherosclerotic lesions. The results of experiments investigating whether the activation of the complement through the alternative pathway is atherogenic or anti-atherogenic are inconclusive yet and showed inconsistent results (255) (264) (265).

Terminal complement complex C5b-9 (“membrane attack complex”)

Complement complex C5b-9 can be detected at increased levels in atherosclerotic lesions. Unaffected arterial wall contains C5b-9 but in smaller amount. It is especially present in the early stage of atherosclerosis, the intimal thickening (266). Later states of atherosclerosis, e.g. fibrous plaques, contain less C5b-9. Co-localization studies showed that C5b-9 co-localizes with many molecules, structures and cells being involved in atherogenesis, e.g. lipid droplets, macrophages, smooth muscle cells, apoptotic cells and cell debris (254) (267) (268). C5b-9 co-localizes with enzymatically modified LDL in the intima, with macrophages and foam cells (268) (269) (270). It is possible that the generation of C5b-9 complexes is triggered by apoptotic cells and that bystander cells die due to insertion of C5b-9 complex into their membrane, maintaining inflammation (270). Depending on its concentration, C5b-9 can have different effects on the affected cells. In higher concentrations C5b-9 complexes are lytic and can cause death of the cell, in lower sub-lytic concentrations, they can change the affected cell’s metabolism (271).

Effects of “sub-lytic” C5b-9 on vascular smooth muscle cells

Vascular smooth muscle cells co-localize with C5b-9 in atherosclerotic lesions (272). The contact with sub-lytic C5b-9 induces several changes in the SMC gene expression, resulting in cell cycle progression and smooth muscle cell proliferation (273), and recruitment of
monocytes by increased production of MCP-1 (274). The MAPK cascades leading to activation of ERK1, JNK1 and p38 pathways are stimulated, resulting in cell cycle activation and cell proliferation. The ERK pathway seems to be most strongly stimulated by sub-lytic C5b-9 and is the pathway primarily responsible for cell cycle activation. Additionally, the phosphoinositide 3-kinase (PI3K) pathway and its dependent Akt pathway are activated by sub-lytic C5b-9, further stimulating cell proliferation of the SMC. The effects of sublytic C5b-9 are supposed to be G-protein dependent, resulting in increased activity of PI3K and ERK (273).

At the same time, sub-lytic C5b-9 “induces IGF-1 and IGF-1 bindings sites in SMCs”, “inhibiting apoptosis of SMCs” (275). The increased expression of IGF-I receptor by VSMC and its stimulation with IGF-I resulted in increased VSMC migration in wound healing assays of murine aorta segments (276). It is still not clear whether these effects of IGF-1 must be considered as risk factors promoting atherosclerosis in the initial phases of the disease or as protective factors improving plaque stability and vasculature elasticity in the later phases of the disease or both (276) (277) (278).

4.1.1.4 Adaptive immunity and atherosclerosis

Atherosclerosis is a chronic inflammatory disease, consequently all components of the immune system are involved, including the T lymphocytes and B lymphocytes, which belong to the adaptive immune system (279) (280) (281). T-cells are regularly found in atherosclerotic plaques (282) (283). In contrast, B lymphocytes are less frequently detected in atherosclerotic plaques from mouse models of atherosclerosis, (284) and scarcely in human atherosclerotic lesions, although regularly present in the diseased vessels’ adventitia (285) (286) (287). Their products, immunoglobulins are regularly found in atherosclerotic plaques derived from human patients (288) (289) or mouse models of atherosclerosis (284). Like in other processes involving inflammation, T helper-cells and cytotoxic T-cells regulate this process by activating other leukocytes directly by cellular interaction or indirectly by release of cytokines, e.g. IFN-gamma (283) (290). Subsets of T helper-cells can reduce the immune response and inflammation, delaying atherosclerosis. T cells have the ability to increase or reduce the severity of inflammatory processes within the intima, which is a key factor in the development of atherosclerosis. By regulating inflammation, T cells additionally influence the stability of atherosclerotic plaques and the probability of plaque rupture and concurrent thrombus formation (291). Antibodies produced by B-cells can either aggravate or attenuate atherosclerosis, depending on their type (292) (293).

4.1.1.4.1 Effects of T lymphocytes in atherosclerosis

The adaptive immune system relies on T cells for activating antigen-presenting cells, e.g. B-cells and macrophages and for killing cells infected with viruses or other intracellular pathogens. B-cells, which have bound their antigen are dependent on T cells to start antibody production. In atherosclerosis, the endothelium enhances its adhesion properties by overexpressing adhesion molecules, e.g. VCAM-1. Consequently, the adhesion and migration of T cells from the blood flow into the intima is increased (294) (295). Another way of entry are the “vasa vasorum”, which supply the blood vessel and are located in the artery’s adventitia (281) (296). Self-antigens and microbial antigens are involved in the activation and expansion of pro-atherosclerotic T lymphocytes, epitopes involved are oxLDL and heat shock protein 60 (Hsp 60) (283) (297) (298). The pathogen *Chlamydia pneumoniae* can release heat shock protein 65 (HSP65), which can cause the activation of T cells which are “auto-reactive” against human HSP60 (299). This may disturb immunological tolerance against human HSP60, resulting in stimulation of CD4+ T cells with auto-immune activity against HSP60 and
increased production of auto-antibodies directed against HSP60 (300) (301). Oxidized LDL (oxLDL) is another antigen frequently recognized by T cells. Around 10% of T lymphocytes located in atherosclerotic plaques recognize it, if presented on MHC II molecules (283). In ApoE-deficient mice, analysis of mRNA extracted from atherosclerotic plaques showed an increased number of transcripts coding for the T cell receptor specific for oxLDL in comparison to controls (302). Atherosclerosis is thus an inflammatory disease T cells participate in. T cells found in an atherosclerotic plaque belong to several subsets, each of it has a defined function in inflammation and atherosclerosis (303). Interestingly, T cell clones which expand locally in progressive atherosclerotic lesions locally do not show increased numbers in the peripheral blood, which indicates that atherosclerotic disease is a local inflammatory disease, which can occur in any artery (304) (305). Most studies investigating the role of specific lymphocyte subsets in atherosclerosis are based on murine models of atherosclerosis and results do not necessarily apply to humans. The two most frequently used mouse models of atherosclerosis are the LDL receptor-deficient mouse (LDLR -/-) and the apolipoprotein E-deficient mouse (ApoE -/-) (306). Like in human atherosclerotic lesions, CD4+ T cells and CD8+ T cells can be frequently found in the atherosclerotic plaques of these mice, which are prone for the development of atherosclerosis (307). Some observations made in mice could be directly or indirectly confirmed in humans while others comparisons are inconclusive.

**CD4 positive T cells (T helper cells)**

The most numerous T cells in atherosclerotic plaque are CD4 positive T cells, which are also known as T helper cells (283) (290). Several subsets of CD4 T helper cells exist, which differ in their spectrum of secreted cytokines. Each of these subsets has a unique function in the inflammatory progress of atherosclerosis (303). T helper cells are present in human atherosclerotic plaques, and in plaques of animal models of atherosclerosis (307). They are even detected in early phases of atherosclerotic lesion formation and promote aggravation of the disease. Most of their subsets have pro-atherogenic or mixed effects. In contrast, the regulatory T helper cells (Treg) have a clearly anti-inflammatory, atheroprotective effect (303).

**Th1 cells**

Most T helper cells present in atherosclerotic lesions belong to the subset Th1, which is promoting inflammation (Figure 4.1.1.4.1) (308) (309). One prerequisite for differentiation of T cells into Th1 cells are “cytokines IL-12 and IL-18”, which are secreted by activated macrophages (310). Th1 cells secrete large amounts of interferon gamma (IFNg) and IL-2, which are pro-inflammatory cytokines (290) (311). The effects of IFNg on atherosclerosis are harmful, it stimulates the phagocytosis of lipids by macrophages and increases the migration of further leukocytes into the atherosclerotic lesion (312). By contact with IFNg, the antigen presenting activity of immune cells and endothelial cells is increased. This is partially caused by upregulation of MHC II molecules on potential antigen presenting cells (313) (314). At the same time, B cells are stimulated to produce IgG2a by IFNg (315). The differentiated Th1 cells also promote differentiation of other naïve T cells into Th1 cells by secreting IFNg and IL-12, further increasing inflammation (316) (317). Several experiments analyzed the effects of IFNg on atherosclerosis. Repeated injections of recombinant IFNg into ApoE-deficient mice resulted in increased lesion size of atherosclerotic lesions (318). Similar effects where caused by administration of IFNg to LDL receptor-deficient mice. Knockout of the IFNg receptor in ApoE-deficient mice reduced the lesion size by 60%, and the plaques had an increased collagen content, indicating higher plaque stability (319). Similarly, mice with LDL receptor-deficiency (LDLR-/-) and IFNg-deficiency developed less atherosclerotic lesions than their counterparts with sole LDL receptor deficiency (320).
The differentiation of Th1 cells as well as their stimulation is caused by IL-12 and IL-18, which are released by activated macrophages which are abundant in atherosclerosis lesions. The effects of IL-12 and IL-18 are strongly pro-atherosclerotic, most probably by stimulating the secretion of IFNγ by Th1 cells. ApoE-deficient mice with IL-12 knockout developed less severe atherosclerosis than their counterpart without IL-12 knockout. The area of the aortic root covered with atherosclerotic plaques was decreased by over 50% (321). The inactivation of IL-18, e.g. by IL-18-binding protein, decreased the development of atherosclerotic plaques and reduced the number of cells in them, while the collagen content was increased. More healthy-appearing vascular smooth muscle cells producing collagen could be found in atherosclerotic plaques formed in IL-18-deficient/ApoE-deficient mice, and atherosclerotic lesion size was reduced by roughly one third (309). The transcription factor necessary for the differentiation into Th1 cells is T-bet, its deficiency also reduces buildup of atherosclerotic plaques in ApoE-deficient mice (322).

**Th2 cells in atherosclerosis**

The role of Th2 cells in the pathogenesis of atherosclerosis is less clear. Upon activation, they secrete IL-4, IL-5 and IL-13. While some effects of IL-4 are atheroprotective, others are pro-atherogenic. On one hand, IL-4 inhibits the development of Th1 cells (323), which secrete the pro-inflammatory IFNγ. In addition, IL-4 alters macrophage differentiation favoring the less damaging M2-phenotype. On the other hand, IL-4 stimulates mast cells, which then exert pro-inflammatory effects leading to protease production within the intima and apoptosis of vascular smooth muscle cells, resulting in the destabilization of atherosclerotic plaques (324) (325).
Expression of the matrix metalloproteinase 12 (MMP-12) within the plaque is induced by IL-4, causing further collagen destruction and tissue damage (326). MMP-12, an elastase, is known to digest components of the arterial wall matrix and is also involved in the development of human aneurysm and atherosclerosis (327) (328) (329). In mouse models of atherosclerosis, IL-4 enhances expression of scavenger receptor A and CD36 on macrophages, increasing the macrophages’ ability to take up LDL (330) (331) (332). When exposed to IL-4, endothelial cells and VSMCs increase production of adhesion molecules like VCAM and of chemokines, e.g. MCP-1, which promote monocyte adhesion and migration (333) (334) (335). Smooth muscle cells can produce more proteolytic enzymes, e.g. MMP-1 after stimulation, which augments the inflammatory process (336). Experimental results are inconclusive whether or under which circumstances IL-4 is predominantly anti-atherosclerotic or pro-atherosclerotic. The transplantation of IL 4 -/- bone marrow to ApoE-deficient or LDL receptor-deficient mice resulted in less atherosclerosis (321) (337). The second cytokine secreted by Th2 cells is IL-5, which is supposed to have anti-atherosclerotic effects by supporting the development of B1 cells (338), which can produce atheroprotective IgM antibodies (339). Transplantation of IL-5-/- bone marrow into LDL receptor-deficient mice aggravated atherosclerosis (340).

**Th17-cells**

These CD4+ T helper cells are still under investigation, and their role in atherosclerosis is not defined. Presumably their effect on atherosclerosis is context-dependent, i.e. dependent on the other cells present in the lesion, their status and their cytokine production (341). Other factors for the effect of Th17 cell activity may be the anatomic location of the atherosclerotic plaque and its histopathological stadium. Th17 cells can be found in human and murine atherosclerotic plaques (342) (343) (344). The differentiation of Th17-cells requires IL-6 and TGF-beta. They influence the process of atherosclerosis by secreting IL-17, which is a pro-inflammatory cytokine activating the inflammatory NF-kB pathway (345). Other effects of IL-17 are increased production of TNF-alpha and IL-1-beta in macrophages (346), and the increased systemic release of GM-CSF with subsequent mobilization of neutrophils into the peripheral blood (347). Animal studies examining the effects of increased or reduced activity of IL-17 or its receptor IL-17RA showed inconsistent results. Madhur et al presented that ApoE-deficient mice, which were also deficient for IL-17 showed less cellularity and IFNg secretion in atherosclerotic lesions, but “plaque burden” was not influenced. From these experiments, it could be concluded that IL-17 increases cellular infiltration of atherosclerotic plaques and stimulates IFNg secretion. Interestingly, the diet had influence on the cytokines secreted by T lymphocytes in the spleen. Animals with high fat diet showed increased production of IL-17 in their spleen (348). Eid et al. observed that IL-17 and IFN-g were able to increase the production of pro-inflammatory IL-6, CXCL8 and CXCL10 by cultured VSMCs derived from human atherosclerotic plaques located in the coronary artery (342). Butcher et al. compared plaque development between ApoE-deficient mice with combined IL-17 deficiency or IL-17 receptor-deficiency and ApoE-deficient mice being fed a Western Diet. In this study, blockade of the IL-17 axis resulted in reduced buildup of atherosclerotic plaques in the aortic root and the aortic arch, but atherogenesis in the thoraco-abdominal aorta was not reduced (349). Contrary results were published by Danazaki et al, who observed an increased growth of atherosclerotic plaques with larger vulnerable plaques in IL-17-deficient/ApoE-deficient mice subjected to a diet with a high fat content (350). In vitro experiments showed that IL-17 can reduce the expression of VCAM on endothelial cells. IL-17 also reduced monocyte adhesion to murine and human endothelial cells in vitro (351) (352). In human carotid plaques, expression of IL-17A is accompanied by increased collagen and actin contents and an increased levels of mRNA coding for collagen and actin (352). Human studies showed that Th17 cells and IL-17 might increase plaque stability by stimulating VSMC to synthesize more actin and collagen (343). A patient study investigating patients after myocardial infarction and stent implantation showed that
patients with myocardial infarction had a higher mortality and increased re-infarction rate if they had low levels of Th17 cells in the peripheral blood (353). In contrast, another study showed that patients suffering from an acute coronary syndrome (ACS) had increased numbers of Th17 cells, cytokines secreted by them, including IL-17 but reduced numbers of regulatory T cells in their peripheral blood (354).

**Regulatory T cells (T reg)**

This subset of T helper cells is able to suppress the immune responses, although this mechanism is not completely understood (355) (356). Their characteristic surface markers are CD4 and CD25, while their development is mainly stimulated by TGF-beta and IL-2 (357) (358). The two main cytokines modifying inflammation released by T reg cells are IL-10 and possibly TGF-beta (359) Another characteristic feature is the transcription factor forkhead box P3 (FoxP3) (359). Upon recognizing their antigen on an MHC receptor, they secrete IL10 and/or TGF-beta, two anti-inflammatory cytokines. Considering their maturation and activation, regulatory T cells can be divided into natural and inducible T reg cells. While the natural T reg cells can be directly activated by self-antigen, the inducible T reg cells only differentiate and are only activated after encountering their specific antigen (355). Regulatory T cells are found in human and murine atherosclerotic plaques but in much smaller number than other T cell subsets (360) (361). Their atheroprotective role was determined by depletion studies, which annihilated Tregs in ApoE-deficient mice by targeting them with anti-CD25 immunoglobulins. As a result of T reg depletion, the subjected mice developed more atherosclerotic plaques (362). One of the T reg cells’ task is to prevent autoimmune reactions by competing with other T cells for the antigen-MHC complexes presented by APC. The activity of other cells can be reduced by increased expression of CTLA-4 on the membrane of Tregs. By release of IL-10 and TGF-beta, inflammatory cells can be inhibited by T reg cells (355).

The “adoptive transfer” of regulatory T cells to ApoE-deficient mice lead to elevated IL-10 production and reduced Th1 activity, accompanied by decreased IFNg production. These animals developed less atherosclerosis (362) (363). Meng et al studied the effects of simvastatin on T reg cells. After administration of simvastatin for six weeks, the atherosclerotic plaques of ApoE-deficient mice contained more regulatory T cells, concomitantly the numbers of mRNA coding for IL-10, TGF-beta and FoxP3 were increased (364). Statins increased the number of regulatory T cells when added to mononuclear cell cultures derived from peripheral blood of human patients, who suffered an ACS. After initiation of simvastatin treatment, T regs were more numerous and showed an enhanced inhibitory activity in comparison to T regs isolated before the start of treatment (364).

In humans, low numbers of Tregs in the peripheral blood are associated with an increased risk of myocardial infarction and other “coronary events” (365). Rohm and Yilmaz examined human vulnerable, unstable atherosclerotic lesions and assessed the presence of different subsets of T lymphocytes. The amount of anti-inflammatory T reg cells was much smaller in unstable atherosclerotic plaques than in stable atherosclerotic plaques. Vice versa, pro-inflammatory T helper cells and cytotoxic T cells were more frequent in unstable and vulnerable lesions than in stable lesions (366).

**CD8 positive T cells (cytotoxic T lymphocytes, CTL, “killer T cells”)**

CD8+ T cells are also termed cytotoxic or cytolytic T cells for their ability to kill targeted cells directly or to induce apoptosis in targeted cells (367). CD8+ T lymphocytes are part of the adaptive immune system, they are only activated by one antigen, which is recognized by their T cell receptor. Another prerequisite for the activation of CD8+ T lymphocytes is the
presentation of the antigen by a MHC type I molecule on the antigen-presenting cell. Upon activation, CD8+ T lymphocytes release several cytokines, including TNF-a and IFN-gamma (368) and begin to proliferate, resulting in the generation of many CD8+ effector T lymphocytes capable of killing cells carrying the antigen. There are several mechanisms, by which targeted antigen-presenting cells can be affected by activated CD8+ T lymphocytes. Targeted antigen-presented cells can be killed by the release of cytotoxic granules containing perforin and granzyme, or by the secretion of Fas ligand (FasL) (369). The cytolytic protein perforin can form transmembrane tubules, which induce a pore in the target cell’s membrane. The existence of these pores alone can be lethal for the cell, as the border between extra- and intracellular space is broken, allowing influx of pro-apoptotic calcium (370). Granzymes, which are pro-apoptotic proteases, can afterwards passively enter the cell through the pore and stimulate apoptosis. Intracellular granzyme B activates caspase-3, which executes the apoptotic process (370). Another mechanism, by which activated CD8+ T lymphocytes can kill other cells is the secretion of Fas ligand. Upon binding of FasL to its Fas receptor on the targeted cell, apoptosis is induced (371) (372). Another pro-apoptotic factor released by CD8+ T lymphocytes is TNF-related apoptosis-inducing ligand, TRAIL (373).

**Involvement of CD8-positive T lymphocytes in atherosclerosis**

This section outlines mechanisms induced by CD8-positive (CD8+) T lymphocytes in the pathogenesis of atherosclerosis (367) (374). CD8+ T lymphocytes are frequently found in human and murine atherosclerotic lesions (282) (375) (376). Their migration into the sub-intima depends on chemokines set free by the inflamed tissue. Their number is highly increased in vulnerable atherosclerotic plaques, which contain thin, fragile caps and large necrotic cores (376) (377). By inducing cell apoptosis and by production of cytokines, CD8+ T lymphocytes promote the inflammatory process of atherosclerosis, which may in part be responsible for the dissolution of fibrous caps, rendering the plaque unstable. In accordance with these findings, CD8+ T lymphocytes are especially numerous in the vicinity of fibrous cap areas (378) (379).

In order to study the effects of CD8+ T cells on atherosclerosis, depletion studies were conducted. ApoE-deficient mice receiving a high-fat diet were treated with antibodies directed against the surface marker CD8+ to eliminate CD8+ T lymphocytes. These mice showed less severe atherosclerosis than their ApoE-deficient controls, which were not depleted from CD8+ T lymphocytes (380). The same general observations could be made in LDL receptor-deficient mice receiving anti-CD8 IgG (381). The effect of cytolytic proteins released from CD8+ T cells was examined by adoptive transfer of mutant CD8+ T cells or wild-type CD8+ T cells to ApoE-deficient mice which had been depleted from CD8+ T lymphocytes. Transferring mutated CD8+ T cells, which do not express perforin, granzyme and TNF-a showed no pro-atherosclerotic effect, while the adoptive transfer of wildtype CD8+ T cells aggravated atherosclerosis. Two cytokines which support the activation of CD8+ T cells and stimulate the synthesis of cytolytic molecules by them are IL-2 and IL-1 (382) (383). Atherosclerosis was reduced in ApoE-deficient mice and LDL receptor-deficient mice, which received antibodies directed against IL-2 and IL-15 (384) (385).

The mechanisms, by which CD 8+ T cells are activated in plaques are diverse. After activation, cytotoxic T cells overexpress several chemokine receptors, which sense chemokines released by the inflamed tissue cells and leukocytes (386) (387). The two most important targets of their cytolytic activity in the atherosclerotic lesion are macrophages and vascular smooth muscle cells (380). The density of cytolytic CD8+ T cells is higher in the vicinity of the thin fibrous cap of vulnerable atherosclerotic plaques, especially in the shoulder region, which is very thin and prone for rupture (388). By inducing apoptosis of vascular smooth muscle cells, the plaque’s stability is reduced, partially due to decreased synthesis of fibrous fibers, which can
stabilize an atherosclerotic lesion. Apoptosis of macrophages is also detrimental. Modified phagocytosed lipids stored inside them are set free and matrix metalloproteinases are released, resulting in more tissue damage and enlargement of the lesion’s necrotic lipid core (329). Thin fibrous caps and large necrotic cores next to CD8+ T lymphocytes are “characteristic features of vulnerable atherosclerotic plaques” (380), which are regularly found in patients with coronary artery disease who have died from sudden cardiac death. Rupture of these vulnerable lesions either exposes the highly thrombogenic surface of the necrotic lipid core to the blood or sets free thrombogenic embolic material, which can cause vessel occlusion resulting in myocardial infarction. Small subsets of regulatory CD8+ T cells exist, the CD25 positive T cells. After immunization of these cells with Apo B-100, atherosclerosis was reduced and cytolytic depletion of macrophages was inhibited (389) (390) (391). Subsets of CD8+ CD25+ T lymphocytes exist not only in mice, but also in humans (392).

4.1.1.4.2 The role of B cells in atherosclerosis

The role of B cells in atherosclerosis is outlined in the following section (393). Antibodies are produced by B cells, making them one of the key players of immunity. B cells are involved in the regulation of atherosclerotic processes by boosting inflammatory reactions locally and secreting antibodies, which can be atheroprotective or atherogenic (393) (394) (395). The different subsets of B cells need to be considered to understand the role of B cells in the pathogenesis of atherosclerosis (Figure 4.1.1.4.2).

**Figure 4.1.1.4.2** The role of B cells in atherosclerosis (from: Srikakulapu and McNamara, B cells and atherosclerosis, Am J. Physiol. Heart Circ. Physiol 312, H1060-H1067, 2017)

**B-1 cells**

Two main subsets of murine B-1 cells exist, B-1a cells and B-1b cells (396).

**B-1a cells**

B-1a cells synthesize antibodies of the IgM isotype. In contrast to B-2 cells, these cells secrete IgM antibodies without being stimulated by a specific antigen (397). Actually, these cells are activated by encountering unspecific triggers. Their antibodies are rather unspecific and are "germ line-encoded", meaning their specificity has not been changed by extensive genetic
rarrangement or selection by antigen-specificity. According to their characteristics, these antibodies are alternatively termed "natural antibodies" (398). Some of these antibodies bind "oxidation-specific epitopes" (OSE), which are located on oxidized LDL (oxLDL) (399), others bind to apoptotic cells (400). Binding of IgM to oxLDL prevents the uptake by macrophages and the generation of foam cells (339) (401). As a consequence, inflammation and progress of atherosclerosis are curbed (393) (402) (403). B-1a cells have a positive influence on the process of efferocytosis, which is the removal of apoptotic cells from tissue - in this case the arterial intima. Apoptotic cells, which are marked by IgM can be more easily degraded by macrophages. Their residues can leave the intima, which can slow down inflammation (404) (405). The human equivalent to murine B-1 cells are the CD20⁺CD27⁺CD43⁺CD70⁺ B cells. The surface marker distinguishing murine B-1a cells from B-1b cells is CD5, i.e. B-1a cells are CD5⁺ in the mouse (406). Taken together, IgM antibodies produced by B-1a cells and the natural IgM antibodies produced by them must be considered protective against atherosclerosis (407) (408) (409). This conclusion is supported by studies from Tsimkas et al. who demonstrated that low levels of IgM antibodies against oxLDL are related to a more severe CAD, and Sjöberg who showed that lower concentrations of IgM directed against phosphoryl-choline-A were associated with an increased risk of ischemic stroke (410) (411).

B-1b cells

In contrast to B-1a cells, B-1b cells are not only activated by unspecific antigen stimuli but also by specific antigens (412). They constitute a distinct B cell subset in mice (413). B-1b cells can react to antigens without involvement of T cells. This stimulation results in the secretion of antigen-specific IgM and IgG3 (414). Following their selection and activation the B-1b cells can transform into memory B-cells, which can act independently from T-cells, a functionality, which has not been identified in B-1a cells (415) (416) (417).

The IgM antibodies secreted by B-1b cells are reactive to OSE (oxidation-specific epitopes) on oxLDL and can be found in the circulation of mice. These antibodies are atheroprotective and attenuate the process of atherosclerosis (412). Concerning the surface markers, they do not carry the surface marker CD5, they are CD5⁻, in contrast to the CD5⁺ B-1a cells. "Adaptive transfer of B1-a cells or B1-b cells into Rag1⁻/⁻ Apoe⁻/⁻ hosts" resulted in high titers of IgM reactive to OSE on oxLDL. The B-cell specific knockout of Id3 in ApoE-deficient mice resulted in reduced "western diet-induced atherosclerosis" (412). The unspecific stimulation of TLR4 by lipopolysaccharides resulted in IgM secretion of B-1a and B-1b cells in vitro, confirming the ability of B-1b cells to be stimulated by unspecific antigens (412). A human equivalent to B-1b cells still has to be identified.

B-2 cells

B-2 cells are "classical B-cells", which are activated antigen-specifically by binding specific antigens to their B cell-receptor and consecutive activation by CD4⁺ T-helper cells. The activation of these cells results in the production and secretion of immunoglobulins, on the long run predominantly IgG (418).

Increased amounts of IgG directed against oxLDL and other modified LDL particles can be detected in atherosclerotic plaques of mice and humans (419) (420). Several experiments and studies showed that their activity promotes atherosclerosis in mice and humans. Due to their capability to produce pro-atherogenic IgG antibodies, B2 cells can be considered the atherogenic subset of B cells (393). This thesis is supported by several animal studies. B-2 cell
depletion studies using anti-CD20 monoclonal antibodies or Baffr-deficient mice showed reduced levels of serum IgG and reduced content of IgG in atherosclerotic plaques. The serum concentration of atherogenic IgG directed against OSE on oxLDL is reduced in these animal models (421). Concomitantly less IgG could be detected in plaques and the progression of atherosclerosis was reduced in Baffr deficient Apo E deficient mice when compared to Apo E deficient controls (422). The same observations of reduced plaque buildup applied to Apo E deficient mice on “Western diet” receiving monoclonal CD20-antibodies (423). The adoptive transfer of splenic B-cells from c57BL/6 mice to B cell-deficient mu-MT ApoE/-/- mice with the same background resulted in "aggravated atherosclerosis" in mice receiving Western diet (293). Strikingly, the amount of T cells in the atherosclerotic plaque was reduced in these studies.

In humans, the serum levels of IgG against oxLDL correlate with CAD in humans, while the serum levels of atheroprotective IgM against oxLDL are inversely correlated to CAD (424). Significantly increased concentrations of IgG directed against oxLDL were measured in patients with systemic autoimmune diseases like SLE and vascular complications in comparison to the same patients suffering from SLE but without vascular complications (425).

The IgG-LDL complexes bind to the Fc gamma receptor of macrophages, granulocytes, B cells and dendritic cells. By binding of these immune complexes to their Fc gamma receptors cells are activated and promote local inflammation (426). Complement can also be activated by immune complexes, potentially aggravating the inflammatory process of atherosclerosis. Mast cells are activated by binding of IgE to their IgE-Fc receptor, their activation promotes inflammation and atherogenesis further (427).

**Regulatory B cells**

Regulatory B-cells are a third group of B cells involved in atherosclerosis. They mainly secrete the anti-inflammatory cytokine IL-10 upon activation. C57BL/6 mice which are IL10-deficient and received chow with high-fat content, had increased generation of fatty streaks (428). Caligiuri et al crossed atherosclerosis-prone ApoE-deficient mice with IL 10-deficient mice and examined that mice deficient for Apo E and IL-10 developed more severe atherosclerosis (429). The overexpression of IL-10 after “adenovirus-mediated gene transfer” by LDLR-deficient mice resulted in reduced atherosclerosis (430). By producing IL-10, Bregs may also induce maturation of Tregs and survival of Tregs, which secrete IL-10 themselves (431) (432).

The mechanisms, by which regulatory B cells can dampen the inflammatory processes in atherosclerosis are not fully understood yet.

### 4.1.1.5 The role of the Renin Angiotensin Aldosterone System in atherosclerosis

Atherosclerosis is a chronic inflammatory disease, which involves the vasculature, the immune system and other key players. Many organs, e.g. liver, kidney and adipose tissue are sources of “soluble inflammatory mediators” and influence the development of atherosclerosis. These include cytokines, chemokines, growth factors and endocrine hormones. Most of the hormones of the renin angiotensin aldosterone system (RAAS) are pro-inflammatory and can maintain or aggravate atherosclerosis. The most important pro-atherosclerotic hormone belonging to this group is angiotensin II.

**Overview of components of the Renin Angiotensin Aldosterone System (RAAS)**

As an introduction to the role of the renin angiotensin aldosterone system (RAAS) in atherosclerosis, the following section gives a brief overview of this system (433) (434). The renin angiotensin aldosterone system consists of several organs, the hormones set free by them, modifying enzymes and target receptors on certain cells and organs (Figure 4.1.1.5).
Angiotensinogen which is the common precursor of angiotensin I and angiotensin II, is expressed by hepatocytes. After leaving the liver and entering the circulation, angiotensinogen is cleaved into angiotensin I by the endopeptidase renin which is secreted into the blood by specialized cells residing in the kidney. These cells are called juxtaglomerular cells and are located in the macula densa between each nephron’s glomerulus and its distal convoluted tubule (DCT). Minor extrarenal sources of renin are derived from the suprarenal gland, uterus, hypophysis and salivary glands. Angiotensin I is cleaved into angiotensin II by the angiotensin converting enzyme 1 (ACE; ACE-1) in the circulation, especially when passing the pulmonary or renal circulation. ACE-1 (ACE) is a zinc metallo-protease which is mainly synthesized by endothelial cells of the pulmonary capillaries, small muscular arteries and blood vessels of the kidney. It exists in an endothelium-bound form and a soluble form. On angiotensin I, it acts as a dipeptidase and cleaves off two amino acids (His-Leu).

The resulting angiotensin II binds to the angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). The most frequent angiotensin receptor in adults is the AT1R. It is responsible for the main effects of angiotensin II (Figure 4.1.1.5).

\[ \text{Angiotensinogen} \rightarrow \text{Renin} \rightarrow \text{Angiotensin I} \rightarrow \text{ACE} \rightarrow \text{Angiotensin II} \rightarrow \text{AT1-Receptor} \rightarrow \text{Vasoconstriction, Aldosterone secretion} \rightarrow \text{Blood pressure increase} \]

*Figure 4.1.1.5 Overview of major components of the renin angiotensin aldosterone system (RAAS).*

In contrast to the AT1R, the AT2R is mainly expressed in fetal tissues. Its expression is massively reduced after birth. Angiotensin II is a main effector of blood pressure regulation. These vascular effects are mainly mediated by angiotensin II-stimulated activation of the AT1R. On the arteries, AT1R leads to vasoconstriction, resulting in increased peripheral resistance. This is the main pathway of increasing blood pressure. In the pituitary gland, it increases secretion of ADH, which promotes the reabsorption of water in the kidney. Angiotensin II-dependent AT1 receptor stimulation also enhances the secretion of aldosterone by the suprarenal gland. Aldosterone increases blood pressure by stimulating the reabsorption of water and sodium in the kidney and by direct vasoconstrictor effects on the arteries.
Some components of the RAAS can modulate and counterbalance the effects of the classical RAAS. This happens mainly on local level within tissues. For instance, ACE-2 converts angiotensin I into angiotensin 1-7 (Ang 1-7), which can induce vasodilation in blood vessels. ACE-2 is up-regulated on tissue level upon ACE-1 inhibition or blockade of the AT1R but not systemically within the circulatory system.

**Angiotensin II and atherosclerosis**

Angiotensin II is one of the main hormones increasing blood pressure and causing hypertension, which is a risk factor for atherosclerosis. Furthermore, angiotensin II promotes atherosclerosis by maintaining inflammation within the arterial wall. It is considered to be the “main pro-atherosclerotic mediator” of the renin angiotensin aldosterone system, RAAS (435) (436). Most systems involved in the pathogenesis of atherosclerosis are stimulated by angiotensin II-mediated AT1 receptor activation. For instance, the expression of adhesion molecules, cytokines, chemokines and growth factors in the arterial wall is up-regulated. Inflammatory mediators like TNF-α, IL-6 and COX-2 increase within the arterial wall. In addition, the adhesion of leukocytes and their migration into the arterial wall are enhanced by increased expression of VCAM, ICAM-1 and P-selectin on endothelial cells. The accumulation of cholesterol is elevated by increased expression of oxLDL receptors (syn. LOX-1 receptors) on endothelial cells. Macrophages are activated, their production of superoxides, metalloproteinase and lipid peroxidases increases. Macrophages themselves contain high amounts of ACE-1. Their capacity of phagocytosis and LDL-oxidation is increased by angiotensin II. AT1 receptor stimulation promotes oxidative stress by higher activity of metalloproteinases and lipid peroxidases, and superoxide generation. As a consequence, nitric oxide (NO) and prostacyclin (PGI2) are destroyed. The complement system is also activated by angiotensin II, which leads to an increased recruitment of inflammatory cells into the arterial wall. In parallel, these recruited inflammatory cells contain ACE and produce angiotensin II themselves. By this mechanism, the local RAS is enhanced and a positive feedback loop is established. Many of the pro-inflammatory effects induced by the binding of angiotensin II to the AT1R result from activation of the NF-κappa B pathway, leading to the activation of kinases. The NF-kappa B pathway is one of the main pathways promoting atherosclerosis, macrophage activation and macrophage survival. (437)

The role of the angiotensin II receptor type 2 receptor (AT2R) in atherosclerosis is still unclear. Their stimulation results also in the stimulation of the NF-kappa B pathway, but it mainly stimulates the activity of phosphatases, in contrast to the AT1R. Upon stimulation of the AT2R endothelial cells produce more bradykinin and their NO synthase activity is enhanced. In vitro studies showed that growth of cultured VSMCs can be reduced by activation of the AT2R. Altogether, AT2R may be counterbalancing the effects of angiotensin II and AT1R stimulation on tissue level (438) (439) (440).

**Angiotensin II AT1 receptor stimulation enhances blood coagulation**

Some of the inflammation mediators regulated by the RAAS can influence coagulation. The capacity for fibrinolysis is reduced, while coagulation is enhanced, promoting thrombosis. These effects are partially caused by decreased expression of the tissue plasminogen activator (tPA) and increase in its inhibitor plasminogen activator inhibitor-1 (PAI-1) (441) by endothelial cells and VSMCs. Platelets are also activated and release thromboxane A2 and PDGF. Through angiotensin II-dependent stimulation of the AT1 receptor, the synthesis of tissue factor in monocytes could be also increased, leading to changed coagulation properties favoring thrombosis (442).
Overall, the activated RAAS system is an established mediator of atherosclerosis, which can be targeted by ACE inhibitors and AT1 receptor blockers.

4.1.2 Diagnosis of stable Coronary Artery Disease

Atherosclerosis can occur in any artery. The process of atherosclerosis is largely similar in all arteries, but the symptoms differ according to the organ affected by the reduced supply with blood. The following chapter gives an overview of general recommendations for the diagnosis and management of patients with stable ischemic heart disease (SIHD) (443).

4.1.2.1 Patient history and physical examination

While taking a patient’s history one must ask for the presence of CVD, stroke and other atherosclerotic diseases in the family. Searching for clues pointing to inherited susceptibility to cardiovascular diseases is essential. The most important symptom of CHD is chest pain which is provoked by physical activity or mental stress resulting in ischemia of the myocardium. Pain due to angina pectoris can also be felt in the upper abdomen, shoulders or jaws. If the level of pain and the pattern how to provoke pain are constant, the term stable angina applies.

If the patient is known to have stable angina, every change in the nature of this pain or the activities causing it must be considered an indicator for changed perfusion of the coronary arteries, possibly by a ruptured atherosclerotic plaque. This condition is called unstable angina (UA) and is also considered as an acute coronary syndrome (ACS). One form of unstable angina is the crescendo-angina with increasing pain, prolonged periods of pain or shorter intervals between phases of pain.

Long-lasting CHD can result in heart failure. Consequently, patients with CHD may show symptoms of heart failure like edema of the lower ankles and lower limbs or short breath.

Excluding differential diagnoses

Several other conditions or diseases can be misinterpreted as angina. These are e.g. pain syndromes of the chest wall, gastro-esophageal reflux disease, pneumonia or cholecystitis. In some cases, the angina, which is caused by ischemia of the heart muscle results from other conditions or diseases. The heart’s oxygen demand can be increased or the supply with oxygen is reduced, both conditions can trigger angina not caused by obstruction of coronary arteries.

4.1.2.2 Resting ECG

If coronary heart disease is suspected, a resting electrocardiography (ECG) is done to allow risk assessment. Electrophysiologic abnormalities in the record can indicate ischemia, arrhythmia or hypertrophy of the heart. Myocardial infarction as a consequence of CHD and prolonged ischemia can also be diagnosed. If abnormalities are present in the resting ECG, the probability of CHD is higher. Patients with stable angina pectoris and an abnormal resting ECG have higher morbidity and mortality rates.

4.1.2.3 Probability estimate

The results of taking the patient’s history, physical examination and resting ECG are evaluated and combined to assess the probability of CHD. Patients with a probability estimate between 20% and 70% have the greatest benefit from further testing by exercise stress ECG. Several
studies have examined the impact of risk factors on the probability of CHD. Important predictors are type of pain, age, gender, abnormalities in the resting ECG, hyperlipidemia, smoking and diabetes mellitus. These findings of earlier studies were confirmed by results of the CASS (Coronary Artery Surgery Study) and analysis of the Duke Databank for Cardiovascular Disease. The pretest likelihood of CHD in patients showing symptoms of CHD can be presented in tables. While CASS data includes only character of pain, age and gender for pretest likelihood of CHD, the Duke Database additionally distinguishes between low-risk patients with no risk factors and high-risk patients with risk factors like hyperlipidemia or diabetes. The presence of abnormal Q-waves or ST-T-wave changes on the resting ECG are increasing the likelihood of CHD in any symptomatic patient.

4.1.2.4 Noninvasive Testing for Diagnosis of CHD – Functional Testing

Functional testing

The aim of functional testing is to detect coronary stenosis which reduces the heart’s supply with blood and oxygen. This is achieved either by increasing oxygen consumption by the myocardium or by decreasing blood flow through the stenotic coronary arteries. Anginal pain and changes in the ECG which is registered simultaneously are indicators of CHD.

Exercise stress testing

If the patient is able to perform many activities of daily living on his own, he should be considered healthy enough to perform functional exercise testing. Exercise testing induces a more physiological stress on the patient. Exercise capacity can be measured, being itself a “very strong prognostic indicator”.

In case of conventional exercise testing the patient has to conduct physical work, e.g. running on a treadmill or cycling on an ergometer while an ECG is recorded simultaneously to detect any abnormalities occurring during the testing. The intensity of exercise increases stepwise, following well established treadmill protocols. The physical work leads to increased myocardial work and oxygen consumption, resulting in a higher demand for oxygen. The blood flow through stenotic coronary arteries cannot be increased sufficiently. Changes in the ECG and anginal pain may develop if ischemia is triggered. It must be pointed out, that coronary stenosis <70% are often not detected by functional testing. The exercise stress must be severe enough to provoke ischemia if a stenosis is present. That is the reason why a “high level of exercise”, reaching “maximal exertion” is the goal.

If maximal exertion could be reached, an exercise ECG showing no abnormalities “excludes obstructive CHD”. If ECG abnormalities or angina pain occur during the exercise testing, a significant or severe CHD is likely. The test results in patients, who show a normal ECG and cannot reach 85% of their “age-predicted maximal heart rate“, or cannot reach highly intense exercise levels must be considered inconclusive, the “estimation of CHD is indeterminate”. “In these cases less intensive treadmill protocols with slower progression can be tried, allowing patients to reach “maximal exercise capacity”. Another alternative is cycling on the ergometer. In case of an abnormal stress ECG, the leads showing electrophysiological changes can hint to the region of the heart affected by ischemia and the vessel most probably responsible. Landmarks for an “ischemic ECG” are horizontal or down-sloping ST-segment depressions at peak exercise”. ST-segment elevation during or after exercise “represents a high-risk ECG consistent with acute ischemia ACS. Stress-ECG can be combined with echocardiography, the ultrasound examination of the heart, to get more “prognostic information that is likely to alter clinical and therapeutic management”, e.g. to show changes in wall motility during the stress
test or if the ECG is inconclusive. Furthermore, exercise stress testing can be combined with other imaging techniques of the heart, e.g. cardiac myocardial perfusion imaging (MPI) by nuclear imaging techniques, CT coronary angiography (CTA) or cardiac magnetic resonance imaging (CMR).

**Pharmacological stress testing combined with cardiac imaging**

Patients not capable to perform exercise stress and patients with “uninterpretable ECG” should perform pharmacological stress testing in combination with cardiac imaging. Two different pharmacological mechanism to provoke ischemia in case of CHD are currently in use. The application of beta-agonistic substances like dobutamin leads to elevated heart rate and increased inotropy. This results in increased oxygen consumption by the myocardium and increased demand for oxygen. The second pharmacological mechanism unmasking CHD by causing ischemia is vasodilation. Substances used for vasodilation are adenosine, dipyridamole or regadenoson. The coronary arteries not or least affected by CHD are able to react and dilate, (coronary steal effect). Pharmacological stress testing is usually combined with ECG and echocardiography (“Pharmacological stress echocardiography”). Pharmacological stress testing can also be combined with other imaging techniques, e.g. CMR.

**4.1.2.5 The ischemic cascade**

“Mismatch of oxygen demand by the myocardium and oxygen supply” leads to a cascade of several changes in the heart muscle and its vasculature. This process is called the “ischemic cascade” (Figure 4.1.2.5). The longer or more severe the ischemia, the more manifestations of ischemia occur. These are vascular dysfunction, perfusion deficits, altered metabolism of cardiomyocytes, impaired function of the heart muscle like diastolic dysfunction, regional wall motion disturbances and global systolic function. Changes in the ECG pointing to ischemia and angina pain are more severe symptoms. If severe ischemia persists it causes necrosis of myocardial tissue and myocardial infarction (443).

![Figure 4.1.2.5 The ischemic cascade](from: Fihn et al. Circulation 126, e354-e471, 2012).
4.1.3 Treatment of risk factors for Coronary Artery Disease

Overview

In this chapter, different approaches to reduce the development or progression of coronary artery disease and other atherosclerotic diseases are summarized according to the recommendation of the AHA and the European Society of Cardiology (443) (444).

The therapy of atherosclerotic diseases, including CAD/CHD, is a long-term treatment and several prerequisites must be met to increase the probability of success. Before initiation of the therapy realistic objectives must be determined. Additionally, the patient must have at least some theoretical background concerning his disease. Educating the patient about the disease itself, its symptoms and its possible consequences is essential for the patients’s commitment to the therapy. The therapy itself includes the reduction of modifiable risk factors, e.g. by lifestyle modification, and the reduction of non-modifiable risk factors, in most cases by medication.

4.1.3.1 Reduction of risk factors of atherosclerosis

The behavioral, modifiable risk factors of atherosclerosis include physical inactivity, overweight, smoking, unhealthy nutrition and psychological stress. These risk factors can be countered by lifestyle modification which should include increasing physical activity, improving nutrition and by smoking cessation. Additionally, stress management and therapy of depression can reduce psychological factors predisposing for coronary artery disease. Alcohol consumption should be limited as excess alcohol consumption increases the risk for coronary artery disease.

The cornerstones of reducing non-modifiable and genetic risk factors of atherosclerosis include lifestyle modification and pharmacological treatment of hypercholesterolemia, hypertension and diabetes.

4.1.3.2 Lipid Management

Lifestyle modification

Aim of the lifestyle modification should be the reduction of LDL-cholesterol (LDL-C) levels in the blood serum. Saturated and trans-fatty acids should be substituted by unsaturated fatty acids or dietary carbohydrates. Reduction of saturated fat and cholesterol intake will result in a lowering of “LDL cholesterol by 10% to 15%”. Consumption of about 2 g phytosterols per day can also lower LDL cholesterol by 10% to 15%. The uptake of over 10 g of viscous fibers per day can result in a reduction of LDL-C levels by 3-5%. Weight loss and physical activity are two other factors reducing LDL-C levels. A weight loss of about 5 kg can lower LDL-C levels by 5-8%. Regular physical activity has no direct effect on LDL-C levels but can support weight loss and a healthy lifestyle.

Pharmacological therapy of dyslipidemia

The cornerstone of pharmacological therapy of increased serum cholesterol levels are statins. They are effective in “primary and secondary prevention of coronary events.”

The Cholesterol Treatment Trialist Collaborators (2010) study “analyzed 26 randomized trials of statin therapy” (445). The mean difference of LDL-C between the statin group and the placebo group was 31 mg/dl. Lowering the serum LDL-C concentration by 40 mg/dl reduced all-cause mortality by 10% and mortality due to coronary events by 20%. The incidence of
“nonfatal myocardial infarction, first nonfatal ischemic stroke and the need for coronary revascularization” could also be reduced by 20%. Interestingly, protective effects depended on the sufficient lowering of LDL-C. Statins proved to be protective against several cardiovascular atherosclerotic events, most probably by lowering LDL-C levels.

The Heart Protection Study (HPS)

The HPS study’s aim was to elucidate the effects of taking high-dose simvastatin (40 mg/d) on the general and cardiovascular outcome in high-risk patients with “coronary heart disease, another arterial occlusive disease or diabetes” (446). The treatment group received simvastatin 40 mg/d while the control group took a placebo. The total mortality rate could be reduced by 13% in the treatment group and the coronary mortality rate could be lowered by 18%. The median concentration of LDL-C could be lowered to 88 mg/dl in the treatment group, while the control group had a median LDL-C of 127 mg/dl.

The HPS is a landmark study proving the protective effects of statins on the cardiovascular system in patients with high risk for cardiovascular events. Statins are highly beneficial for cardiovascular high-risk patients.

The TNT (Treating to New Targets) Trial

The TNT study compared the effects of high-dose statin therapy (80 mg atorvastatin/d) with regular-dose statin therapy (10 mg atorvastatin/d) on patients with “clinically apparent” CHD and LDL-C levels above 130 mg/dl (447). The mean LDL-C was 77 mg/dl in the high-dose group and 101 mg/dl in the low-dose group. Patients in the high-dose group had a 20% lower risk to reach a “composite cardiovascular endpoint”. The morbidity due to cardiac death was reduced by 20% in the high-dose group. At the same time, all-cause mortality was not influenced.

The IDEAL Study

The IDEAL trial included only patients with a history of myocardial infarction, who were randomly assigned to an intensive statin therapy by taking 80 mg/d atorvastatin or to a statin therapy of moderate intensity by taking simvastatin 20 mg/d (448). The mean level of LDL-C was 104 mg/dl in the participants receiving moderate intensity therapy with simvastatin, and 81 mg/dl in participants assigned to the intensive therapy with atorvastatin. In the intensive therapy group, a “non-significant trend toward reduction” of a primary cardiovascular composite endpoint was observed. This composite endpoint included coronary death, nonfatal myocardial infarction (MI) and cardiac arrest. Significant reductions were observed in secondary endpoints, including nonfatal myocardial infarction and coronary revascularization.

The conclusion of the IDEAL Study suggests that high-risk patients can benefit from a more intensive cholesterin-lowering therapy, and intensive lowering of LDL-C in patients with stable CHD is important.

The IMPROVE-IT Study

The IMPROVE-IT Study further confirmed that a highly intensive cholesterol-lowering therapy is of additional benefit for high risk patients compared to a moderately intensive LDL-lowering therapy with simvastatin (Figure 4.1.3.2) (449).

Notably, the more intensive LDL-cholesterol lowering was achieved by adding ezetimibe to a statin therapy for treatment of patients after acute coronary syndromes. The IMPROVE-IT investigators found an absolute risk reduction of 2% in the treatment arm with intensive
lowering of LDL-cholesterol to levels below previous targets. The authors conclude that the lower the LDL-cholesterol the better is the cardiovascular outcome (Figure 1.3.2.1) (449).

Clinical guidelines implemented these outcome data from clinical studies and defined therapeutic guidelines for the lowering of LDL-cholesterol. The ATP III guidelines were still working with borders and target values (450). The new guidelines ATP IV abandoned target values and simply recommend a dose of a statin to achieve a high intensity or low intensity treatment (451).

![Figure 4.1.3.2] Data from LDL cholesterol lowering trials with statins ± ezetimib (IMPROVE-IT study) show a direct relationship between the change in LDL-Cholesterol and clinical benefit (449).

For secondary prevention of CAD, ATP III recommended the following target LDL-C values:
- LDL-C target <100 for proven CAD, and high cardiovascular risk
- LDL-C target <70 for very high cardiovascular risk: multiple major risk factors, e.g. diabetes mellitus, severe and poorly controlled risk factors, multiple risk factors for metabolic syndrome.

The new ATP IV guidelines recommend a reduction from baseline. A high dose is recommended as long as tolerated.

4.1.3.3 Blood pressure management

Blood pressure management follows evidence-based guidelines, which are summarized in the following chapter (18) (19).

Lifestyle modification

The first step in the treatment of hypertension are lifestyle modifications. Patients should reduce sodium intake and modify their nutrition by increasing the share of vegetables and fruits to reduce blood pressure. Normalization of body weight and reaching a BMI below 25 kg/m²
can reduce blood pressure. Losing 10 kg of body weight can result in a decrease of blood pressure between 5 mm Hg and 20 mm Hg. Physical activity is also recommended to lower blood pressure. If the effects of life style modification are not sufficient enough to lower blood pressure below 140/90 mm Hg in uncomplicated cases or below 130/80 mm Hg in diabetics or patients with chronic kidney disease, pharmacological anti-hypertensive therapy must be started.

**Pharmacological therapy of hypertension**

Many placebo-controlled trials have shown that the reduction of diastolic blood pressure by 5 or 6 mmHg can lead to pronounced decrease of cardiovascular mortality, stroke could be reduced by 40% and coronary events could be reduced by 20%. Similar positive effects had the reduction of systolic blood pressure by 10-20 mmHg in older adult patients with isolated systolic hypertension in the treatment group receiving antihypertensive medication. According to the “The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure” (JNC7), the blood pressure should not exceed 140/90 mmHg in case of “uncomplicated hypertension” (18). For patients with higher cardiovascular risk, like patients with diabetes and patients with chronic kidney disease, blood pressure should not be higher than 130/80 mmHg. Patients with stable CHD might benefit from even lower “blood pressure targets”.

The ACCORD study compared the two systolic blood pressure targets of 120 mmHg and 130 mmHg in patients with type 2 diabetes mellitus. The reduction of target blood pressure to 120 mmHg had no positive effect on endpoints (452).

In contrast to the ACCORD study, the SPRINT study was performed with patients without diabetes mellitus mortality (21). The SPRINT study compared blood pressure lowering to two different target values: intensive blood pressure lowering to a target value of 120 mmHg and low intensive therapy to a target value of 130 mmHg. The SPRINT study found a significantly decreased cardiovascular and overall mortality of the intensive blood pressure lowering therapy to a target value of 120 mmHg. These data will be most likely implemented in upcoming treatment guidelines for antihypertensive treatment.

**4.1.3.4 Diabetes management**

Diabetes mellitus is a well-defined and important independent risk factor for the development of cardiovascular disease (453). According to recent data, the relative risk for individuals with diabetes to die from cardiovascular disease is 1.7 fold higher in adults >18 years compared to persons without diabetes mellitus (454).

**Treatment of patients with diabetes mellitus and cardiovascular outcome**

In the UKPDS-33 study, intensive glycemic control of patients with type 2 diabetes mellitus showed to reduce microvascular complications, but cardiovascular events could not be reduced (455). A secondary study, i.e. UKPDS-34, revealed more details concerning the anti-diabetic medications being used in the three treatment groups. Metformin proved to be especially beneficial, but sulfonylurea was not associated with any improvement concerning endpoints of the study. Patients taking metformin had a lower median HbA1c value (7.4%) than patients who were “only” adhering to diabetic diet (8.0%) and were not pharmacologically treated (456). Notably metformin was responsible for beneficial effects on the cardiovascular system and reduction in endpoints, not sulfonylurea. Metformin was able to reduce the HbA1c more sufficiently, patients taking metformin had a median HbA1c of 7.4%, patients adhering to conventional therapy had a median HbA1c of 8.0%. Of even greater importance was the finding
that patients in the metformin group had a risk reduction of 32% in reaching one or more diabetic endpoints. In addition metformin-treated patients showed less weight gain compared to insulin and sulphonylurea (456).

In order to elucidate whether a stricter control of HbA1c results in less cardiovascular events, the ADVANCE trial compared intensified anti-diabetic treatment with a target HbA1c of 6.5% with regular anti-diabetic treatment aiming at a target HbA1c of 7%. In the intensive treatment group, microvascular complications could be significantly reduced. Macrovascular events (nonfatal myocardial infarction, nonfatal stroke, overall cardiovascular mortality) were not reduced in the intensive treatment group (457).

The ACCORD study’s aim was to answer the question whether the further reduction of the HbA1c-level below 6.0% by means of intensified anti-diabetic therapy would be more beneficial than regular diabetes therapy aiming at a HbA1c between 7% and 8%. The study continued for over three years, “major cardiovascular events” like non-fatal MI and nonfatal stroke, were not reduced. Surprisingly, the all-cause mortality was increased in the intensive therapy group by 22% (458).

As a consequence of these study data, the goal HbA1c should be set according to the patient’s risk profile. Young patients and patients with a short history of diabetes benefit most from a HbA1c below 7% because microvascular and macrovascular complications of diabetes can be prevented with higher probability by strict control of blood sugar. Patients not capable to follow the more complicated scheme and recommendations to achieve strict blood sugar control have an increased risk of drug adverse effects, e.g. hypoglycemia. In these patients of high age, with comorbidities, cognitive impairment or long lasting diabetes and its complications a HbA1c value from 7% up to 8.0% is tolerable.

Many patients with diabetes have other risk factors for cardiovascular diseases. In frame of the metabolic syndrome, this can be dyslipidemia, overweight and/or high blood pressure. Taking these risk factors into account, treatment of all risk factors by behavioral changes and multiple medications is essential. The Steno-2 study proved that intensive treatment with multiple drug combinations targeting diverse risk factors combined with behavioral modification resulted in a decreased cardiovascular and overall mortality (459).

### 4.1.4 Pharmacotherapy of atherosclerosis

In frame of this chapter, I will present drugs preventing or reducing the progress of atherosclerosis.

#### 4.1.4.1 Statins

Statins are the most important group of drugs for lowering LDL-cholesterol plasma levels.

**Mechanism of action of statins**

Statins inhibit the enzyme HMG-CoA-reductase and therefore are also named HMG-CoA reductase inhibitors (460). This enzyme transforms acetyl coenzyme A into mevalonate, which is the biochemical precursor of cholesterol. HMG-CoA reductase catalyzes the rate-determining reaction in cholesterol synthesis, a process taking primarily place in the liver. After inhibition of the endogenous cholesterol synthesis by the statin, the hepatocytes increase their expression of LDL-receptors. These receptors bind cholesterol-loaded LDL particles which pass them in the capillary blood flow. The bound LDL-particles are then removed from the blood by receptor-mediated endocytosis, resulting in decreased plasma cholesterol levels. At
the same time statins reduce the synthesis of LDL particles and VLDL particles by the liver, further reducing cholesterol concentration in the plasma. Triglyceride levels are also slightly reduced by statins due to reduced synthesis of VLDL particles which contain higher amounts of triglycerides. Statins have several “pleotropic” effects on other physiological systems besides lipid metabolism (lipid-independent effects). These include effects on hemostasis, vascular function and regulation of inflammation. They even have plaque-stabilizing effects on atherosclerosis. Statins have many beneficial, synergistic effects on factors promoting and maintaining atherosclerosis.

Clinical use of statins

The primary use of statins is the reduction of LDL-cholesterol to reduce the cardiovascular risk in individuals to prevent cardiovascular disease or aggravation of a diagnosed CVD like stroke, myocardial infarction or coronary heart disease. Dyslipidemia (hypercholesterolemia) is involved in atherosclerosis, leading to stroke, coronary heart disease, carotid stenosis and other diseases. Statins are the most effective class of drugs for controlling plasma cholesterol. They can be used to treat multi-factorial dyslipidemia and genetic disorders of the lipid metabolism, e.g. familial hypercholesterolemia. The first statin introduced in regular clinical use was lovastatin in 1987 (460).

Types of statins and basic pharmacology

Statins can be categorized into naturally derived statins and synthetic statins (461). Although all statins share the same mechanism of action concerning the HMG-CoA reductase, they differ slightly in their pharmacological properties. The main excretion of most statins is hepatic, about 90% of them is excreted with the bile. Pravastatin is an exception with 60% being excreted renally. Statins are administered once daily, preferably in the evening because hepatic cholesterol synthesis peaks in the night. Statins with very long half-life, like atorvastatin, can be administered at any time. To prevent side effects, patients with hepatic or renal impairment or disease should be treated with lower doses under strict medical control. Pregnant women should not take statins if avoidable.

Statin effects on plasma lipids and lipoproteins

Pharmacotherapy with statins can lower total cholesterol (TC) and LDL-cholesterol (LDL-C) plasma levels by up 50 to 60%. Triglycerides can be lowered by 30%, the maximum effect being achieved in individuals with high triglyceride levels. The percentage of cholesterol reduction varies between individuals and depends on the statin used. The statins with the highest efficacy are rosuvastatin, atorvastatin and simvastatin (462). The reduction of LDL-C is dose-dependent. Typically, doubling of the lowest starting dose of a statin results in additional 6% of LDL-C reduction. Statins begin to have an effect on plasma LDL-C within hours after administration, the full effect on plasma lipids is reached within 4-6 weeks after onset of therapy. In agreement with the concept of evidence-based medicine, it is desirable to choose statins for therapy which proved their beneficial effects in clinical trials. The lipid-lowering effects of statins remain unchanged even after year-long therapy. After ending statin therapy, LDL-C level return to pre-treatment levels quickly. In most cases, long-term reduction of LDL levels is the aim, consequently statins must be taken life-long.

Effects of statins in clinical trials

Several major clinical trials concerning the beneficial effects of statins on primary and secondary prevention of CVD have been conducted. They all showed that statins can protect patients with coronary heart disease, diabetes, peripheral artery disease, stroke from
cardiovascular events and revascularizations (462) (463). But not only patients with pre-existing CVD benefit from statins, individuals without pre-existing CVD have their risk for CVD reduced. Morbidity and mortality due to cardiovascular disease could be reduced by up to 30% in the statin therapy groups. More recent studies showed that high-dose statin therapy is more efficient in lowering CVD morbidity and mortality than low-dose statin therapy, especially in patients with pre-existing CVD (Figure 4.1.4.1-1 and 4.1.4.1-2). Statin therapy had positive effects on individuals of both genders, all races and all affected age groups. Current studies show that the percentage of LDL reduction is critical for successful therapy (Figure 4.1.4.1-1 and 4.1.4.1-2).

**Figure 4.1.4.1-1** Meta-analysis of LDL-C levels of trials, which compared high-dose (Intensive) to standard-dose (Standard) statin therapy (image adapted from: Cannon et al., J. Am. Coll. Cardiol. 48, 438-445, 2006).

**Figure 4.1.4.1-2** Data from individual trials and a pooled data analysis document a significant (16 %) reduction in coronary death and myocardial infarction after high-dose statin therapy (image adapted from: Cannon et al., J. Am. Coll. Cardiol. 48, 438-445, 2006).

According to these data, recent guidelines issued by the ESC/EASPR in 2016 recommend to lower LDL-C in individuals with very high cardiovascular risk to < 70 mg/dL or aim at a reduction of at least 50% from baseline values, if the baseline value is between 70 and 135 mg/dL (464).

**Side effects of statins**

Statins can have a wide range of side effects, but these side effects are infrequent and only a few of them tend to be severe. Possible common side effects include disturbed bowel function,
nosebleeds, non-allergic rhinitis, headache, muscle pain and hyperglycemia. Rare side effects are medicine-induced hepatitis, skin rash, sleep problems, dizziness and peripheral neuropathy (465).

**Statin-induced myotoxicity and rhabdomyolysis**

By blocking the HMG-CoA reductase and by reducing the availability of cholesterol, statins change cholesterol metabolism. Myocytes and hepatocytes are affected most by these changes. Damage and death of myocytes can cause inflammation and myositis, which at its mildest form causes only muscle pain. In few cases, myositis can become very severe and leads to necrosis of many myocytes, this process is called rhabdomyolysis (466). Large amounts of myoglobin are set free into the blood and can cause life-threatening acute renal failure. Rhabdomyolysis occurs in less than 0.2% of individuals taking statins. Co-medication with certain drugs increases the risk of rhabdomyolysis significantly (erythromycin, nicotinic acid, fibrates, ciclosporin, antifungal agents). Some statins are metabolized by hepatic CYP450, interacting with drug metabolism of other drugs like anticoagulants and diltiazem. Co-medication with these drugs can result in increased plasma levels of the statin and the other drugs metabolized by CYP450, increasing the probability of side effects. In case of muscle pain, the dosage of the statin must be reduced or the statin used must be changed.

**Treatment control of statin therapy**

After the first six weeks of treatment with a statin, the success of the lipid-lowering therapy and the absence of unwanted side effects is controlled. Lipid levels and liver enzymes are controlled. Fasting plasma lipid levels including total cholesterol (TC), LDL-cholesterol and triglycerides are measured to determine the cholesterol-lowering effect of the statin therapy. If the percentage of LDL-cholesterol decrease caused by the statin is not sufficient, the statin therapy can be intensified by increasing the daily dose up to the limit, if tolerated. A follow-up blood test after changing the therapy is essential.

Liver enzymes should be measured simultaneously to detect a possible elevation, possibly caused by a statin-induced hepatitis or myositis. In case of muscle pain, the creatine kinase should be measured, increased values point to a myositis. In case of increased liver enzymes, creatine kinase and/or clinical symptoms, like muscle pain, the statin therapy must be reconsidered. After statin therapy has been successfully adjusted, blood tests determining cholesterol levels and liver enzyme levels are conducted once a year.

### 4.1.4.2 Use of ACE Inhibitors and ARBs in atherosclerosis

**Animal models**

ACE inhibitors and angiotensin II receptor blockers (ARBs) exert protective effects on the arterial wall not only in animal models of hypertension but also in animal models of atherosclerosis (467) (468) (469) (470). Cells involved in the process of inflammation are regulated by them, resulting in anti-atherosclerotic effects. Partly their protective effects are based on their anti-hypertensive effects, partly on direct protective effects exerted in the arterial wall itself. Endothelial cells are protected by both mechanisms. The reduction of elevated blood pressure protects them from increased shear stress. At the same time ACE inhibitors and ARBs reduce endothelial dysfunction also independently from blood pressure. Migration of macrophages and other leukocytes into arterial walls and their phagocytic activity are reduced, the endothelium expresses less adhesion molecules and the inflammatory process is slowed down. Fitting into this scheme is the observation that ACE inhibitors directly inhibit the expression of MCP-1, while angiotensin II itself increases it. Possibly due to reduced influx
and activity of leukocytes and their enzymes oxidative stress in the arterial wall is reduced by inhibition of the RAS, improving the endothelium cells’ functioning. Several animal studies showed that ACE inhibitors in therapeutic dosage reduced the development of atherosclerosis. Other anti-hypertensive drugs reducing blood pressure by similar amount did not show anti-atherosclerotic effects. In some studies, “ACE inhibitors reduced atherosclerosis without altering blood pressure”. Angiotensin receptor blockers showed to be equally effective than ACE inhibitors in reducing hypertension and atherosclerosis in several animal models.

**Clinical trials of ACE inhibitors and ARBs**

There is unequivocal evidence obtained by randomized, placebo-controlled clinical trials that ACE inhibitors decrease the mortality after myocardial infarction (STEMI). A meta-analysis of clinical trials conducted by Gornik and O’Gara in 2004 documented the highly significant reduction in mortality by treatment with an ACE inhibitor after myocardial infarction (Figure 4.1.4.2).

![Figure 1 - Figure 4.1.4.2 A meta-analysis of long-term trials, which documented a highly significant decrease in mortality after myocardial infarction by treatment with an ACE inhibitor (adapted from: Gornik H, O’Gara PT. Adjunctive Therapy. In: Manson JE, Buring JE, Ridker PM, Gaziano JM, editors. Clinical Trials in Heart Disease, a Companion to Braunwald’s Heart Disease. Philadelphia: Elsevier/Saunders; 2004: 109-128)](image)

Anti-atherosclerotic properties of ACE inhibitors were detected in the late 80s and early 90s of the last century although the exact reasons where still unclear. The “concept of the cardiovascular continuum” concerning CVD was established in the early nineties. This scheme proposes a sequence of pathophysiologic changes leading from endothelial dysfunction over atherosclerosis to target organ damage and finally to clinical syndromes, e.g. myocardial infarction or stroke. In agreement with this concept, the first effect of ACE inhibition to be investigated was the influence on endothelial dysfunction. The first investigations showed positive effects of ACE inhibition on endothelial dysfunction, and the TREND study confirmed these effects on the endothelium of coronary arteries. Similar beneficial effects of ARBs on the endothelium could be detected by later studies, after the ARBs had been introduced in the late nineties (471).
Depending on the chemical structure of the ACE inhibitor, they can inhibit soluble ACE or tissue ACE to a varying extent. The BANFF study showed that enalapril, an “ACE inhibitor with lower activity on tissue level”, did not influence endothelial dysfunction (472). The inhibition of plasma ACE is primarily responsible for blood pressure reduction, while the inhibition of ACE on tissue level results in reduction of inflammation, fibrosis and hypertrophy of affected tissues. The capability of the ARB telmisartan and the ACE-inhibitor ramipril to relieve endothelial dysfunction of the renal circulation was compared in the TRENDY study and showed no significant differences (473). Ramipril shows high ACE inhibiting activity on plasma and tissue level, in contrast to enalapril. Clinical parameters to measure endothelial function is measuring the acetylcholine-stimulated blood flow in the forearm. Functional endothelium will show a stronger reaction towards contact with acetylcholine than dysfunctional endothelium.

The anti-inflammatory effects of ACE inhibition or AT1R blockade in patients were explored by several studies measuring the serum “levels of inflammatory markers in different diseases”. Both mechanisms reduced the serum concentration of several inflammatory cytokines and markers. Studies exploring the progression of atherosclerosis under therapy with ACE inhibitors were contradictory, but most studies support the thesis that ACE inhibitors inhibit the progression of atherosclerosis and the growth of atherosclerotic lesions. The LIFE study evaluated the effects of AT1R blockade by the ARB losartan on the progression of atherosclerosis and showed that losartan “induced a regression of the carotid artery hypertrophy in hypertensive subjects” (474). Studies showed that ACE inhibitors are superior to other anti-hypertensive drugs in the pharmacological therapy of CVD with increased activity of the RAS, like congestive heart failure or coronary artery disease in combination with left ventricular dysfunction. Studies investigating whether ARBs could achieve the same effects proved similar treatment effects.

The SAVE and SOLVD studies, which investigated the effects of ACE inhibitors in patients with left ventricular dysfunction, resulted in reductions of mortality rate and reduced risk of ischemic events in the treatment group (475) (476). The SAVE study showed that the effect of ACE inhibition by captopril was independent of the degree of left ventricular dysfunction (475). The effectiveness of the RAS-blockade by ACE-inhibitors in patients without left ventricular dysfunction could be proved by the HOPE study (477). Risk reduction of 22% for reaching a composite endpoint including cardiovascular death, myocardial infarction or stroke could be achieved by therapy with ramipril in patients with high cardiovascular risk. The EUROPA studies confirmed the findings of the HOPE study (478). For comparison, the PEACE study, which enrolled patients with stable coronary artery disease and a lower cardiovascular risk, did not show significant differences between treatment and placebo groups (479).

To compare the beneficial effects of ACE inhibitors and ARBs and to investigate the effects of treatment with both drugs, the ONTARGET study was conducted (480). The effect of ACE inhibitors and ARBs on primary outcomes was similar, both drugs showed the same effectiveness in preventing primary outcomes (death due to cardiovascular causes, myocardial infarction, stroke, hospitalization for heart failure). ACE inhibitors seemed to be more suitable for MI prevention, while ARBs seemed to be more suitable for prevention of stroke. ARBs had less side effects than ACE inhibitors. The combined administration of ACE inhibitor and ARB showed no beneficial effects, but an increased rate of adverse effects resulted. The combination therapy can be recommended in cases of severe heart failure and pre-treatment with beta-blockers.
4.1.5 Emerging approaches for the treatment of hypercholesterinemia and atherosclerosis

Statins are not tolerated well by some patients. The most common adverse effect is the statin-induced myositis, which causes muscle pain. SAMS (statin-associated muscle symptoms) is the most common and mildest type of statin induced myositis, but the prevalence among patients taking statins is high (7%-29%). Much fewer patients experience statin-associated myopathy with increase of creatine kinase (CK). Statistics show that over 75% of patients discontinue the statin therapy within the first two years of treatment. In case of complete intolerance to statins using other cholesterol lowering drugs to reduce LDL-C levels is possible but the benefit of treatment with non-statins has not been confirmed by studies. In case of SAMS, dosage of statins must be reduced until tolerated and can then be combined with non-statin lipid lowering medications. If increase of statin dosing is not tolerated or not effective in uncontrolled hypercholesterolemia, the same strategy applies. The IMPROVE-IT study showed that ezetimibe is the most effective “classical” non-statin drug lowering LDL-C to be combined with statins, if needed (449).

Until a short time ago, statins where the only drugs to be proven to reduce LDL-C, and to reduce morbidity and mortality due to CVD. There was no suitable and approved alternative choice available for patients not tolerating statins. Research focused on developing cholesterol-reducing drugs with a new mode of action. Several new lipid-lowering drugs have been introduced over the last decade.

4.1.5.1 PCSK9 Inhibitors

By the year 2003, the function of proprotein-convertase subtilisin-kexin Type 9 (PCSK9) in cholesterol metabolism had been elucidated and mutations in PCSK9 were found to cause autosomal dominant hypercholesterolemia (481).

Inhibition of this small protein seemed to be a possible way of reducing plasma levels of LDL-C (482). Since then two human monoclonal antibodies have been developed, which entered phase III clinical trials. After they had completed several phase III trials successfully, these antibodies have been approved as therapeutic agents by the FDA and the EMA in 2015.

Overview PCSK9 function

PCSK9 is a protein which influences the density of LDL receptors on the cell membrane post-transcriptionally. It is synthesized by hepatocytes and secreted into the circulation. PCSK9 then binds to LDL receptors and triggers their internalization and complete degradation in lysosomes. PCSK9 is then set free again and can act on further LDL receptors. Monoclonal antibodies directed against PCSK9 inhibit its binding to the LDL receptor, resulting in less degradation and improved removal of LDL-C from the circulation.

4.1.5.2 PCSK9 inhibitors approved for clinical use

PCSK9 inhibitors, which were approved for clinical use, are the two antibodies Alirocumab (Praluent®) and Evolocumab (Repatha®). Both are monoclonal, fully humanized therapeutic antibodies, which have been approved for clinical use. They have proven their effectiveness in lowering LDL-C levels in several clinical studies (483) (484). In addition, clinical data show beneficial cardiovascular outcome data of long-term treatment with evolocumab (485).

The route of administration is subcutaneous (SC) injection, which can be conducted by the trained patient himself. Two injection schemes, once monthly and twice monthly, are
established. The higher the injected dose, the longer lasts the LDL-C lowering effect. These antibodies are normally well tolerated and showed in general only weak side effects. Depending on the circumstances their efficacy to lower LDL-C levels is between 30% and 60%.

PCSK9 inhibitors are often combined with statins to reduce LDL-C levels further in patients, who do not tolerate an increase of statin dosage or have insufficient LDL-C reduction under maximal dosage of statin. In familiar hypercholesterolemia (FH), the addition of PCSK9 inhibitors to a pre-existing statin therapy proved to be superior to increase of statin dosage. Alirocumab and evolocumab have been approved for the therapy of heterozygous FH and uncontrolled hypercholesterolemia in atherosclerotic cardiovascular disease. Evolocumab has additionally been approved for the treatment of homozygous FH. It must be emphasized that therapy with PCSK9 inhibitors is only approved as adjunct therapy to dietetic therapy and maximally tolerated statin therapy.

The humanized antibody bococizumab has been withdrawn from clinical phase III trials after preliminary study results showed that no additional advantages could be expected in comparison to the approved drugs, alirocumab and evolocumab (486). In addition, the development of antidrug antibodies directed against the humanized but not fully human antibody bococizumab was observed in a large number of patients (487).

4.1.5.3 Microsomal triglyceride Transfer protein (MTTP) inhibitor

The microsomal triglyceride transfer protein (MTTP) is essential for assembly and secretion of Apo B-containing lipoproteins in the liver and other gastrointestinal organs.” Lorlatinapide is the only MTTP-inhibitor currently available for human use. It has been approved by the FDA and the EMA for treatment of homozygous familial hypercholesterolemia (FH). Lorlatinapide is administered orally. Limiting are gastrointestinal side effects like diarrhea, vomiting and nausea, but also liver toxicity, which occur frequently (488).

4.1.5.4 Antisense oligonucleotide Mipomersen

Another method to lower LDL levels is to reduce the expression of apolipoprotein B (ApoB), which is an essential component of all cholesterol-containing lipid particles like LDL and VLDL. The antisense oligonucleotide mipomersen binds to ApoB-mRNA and prevents the translation of the ApoB-mRNA, resulting in decreased expression of ApoB (489). The oligonucleotides are injected subcutaneously (SC). Mipomersen can reduce LDL-C levels by 25% to 40% and is only approved for the therapy of homozygous FH if other therapies are not sufficient. Side effects are frequent and include flu-like symptoms and reactions at the injection site. In 10% of patients the liver enzyme ALAT increase to a level 10 times as high as the normal value (489).

4.1.5.4 Novel HDL-targeted therapy approaches

Another concept to reduce the growth of atherosclerotic plaques are HDL-targeted therapies. Their aim is to increase the amount or to improve the function of circulating HDL-C, which is supposed to prevent atherosclerosis by reducing the deposition of LDL-cholesterol in the arteries. So far only few clinical studies could confirm a positive effect of selected drugs increasing HDL. Therapeutic and clinical guidelines do not include target HDL values anymore. The only target for therapy of atherosclerotic vascular disease is LDL-C.
**Cholesteryl ester transfer protein (CETP) inhibitors**

The transfer of cholesterol esters from HDL particles to the lipoprotein ApoB is mediated by CETP. Reducing the transfer of cholesterol esters from HDL particles to lipoprotein ApoB by inhibiting CETP increases cholesterol content of a special subset of large HDL. Two small-molecule CETP-inhibitors are dalcetrapib and torcetrapib. Although both of them were able to increase HDL-C levels significantly in clinical studies, they did not reduce cardiovascular morbidity or mortality or even increased mortality in case of torcetrapib (490).

**Apo A-I mimetics and their phospholipid-HDL complexes**

Apo A-I is one of the most important lipoproteins mediating the efflux of cholesterol from cells. Apo-A-I mimetics are reconstituted with a disc-shaped subgroup of HDL particles to allow release and binding of cholesterol to the HDL. These apolipoproteins, e.g. POPC-Apo A-I-HDL, mediate the efflux of cholesterol from cells, especially from macrophages, and bind the released cholesterol. These effects are partially dependent on ABCA1, but non-specific pathways independent from ABCA1 exist. Patients with Tangier’s Disease lack ABCA1 and suffer from severe atherosclerosis. The infusion of POPC-Apo-A-I-HDL can significantly reduce atherogenesis in these subjects. A more effective variant of Apo-A-I is the so-called Apo AI-“Milano”, which carries a point mutation. Recombinant Apo A-I-“Milano” reconstituted with HDL showed to reduce the size of human coronary atherosclerotic plaques in small studies (491). Further research is conducted to confirm these results.

**Conventional drugs increasing HDL-C levels**

Statins, nicotinic acids and fibrates are drugs known to increase HDL-C levels. Most clinical studies could not confirm beneficial effects on cardiovascular outcomes attributed to these changes of HDL. If combined with statins, the fibrate gemfibrozil reduced overall and cardiovascular mortality in several studies (492). Whether this beneficial effect is due to the HDL-increasing properties of gemfibrozil is not known (see comment above).
4.2. Scientific goal of PhD research project “Microarray gene expression profiling reveals antioxidant-like effects of angiotensin II inhibition in atherosclerosis”

The major treatment approach against atherosclerosis targets hypercholesterolemia, as statins do. ACE inhibition of the angiotensin II system is one of the few alternative approaches that is anti-atherosclerotic without LDL-cholesterol lowering. Elucidation of mechanisms triggered by Ang II inhibition could thus reveal previously unrecognized pathomechanisms and targets for therapy. Our previous study has shown that treatment with Ang II inhibitors targets pro-inflammatory immune cells. In addition, it is known that angiotensin promotes ROS formation. The goal of my studies was to identify ROS-dependent and ROS-independent effects of Ang II inhibition. We used the prototypic ACE inhibitor captopril as an Ang II inhibitor and Apo E deficient mice as a model of atherosclerosis. To identify specific ROS-inhibition mediated effects we performed treatment with the antioxidant vitamin E. Whole genome microarray gene expression profiling was performed to identify genes regulated concomitantly or differentially by treatment with captopril or vitamin E in comparison to control animals.

The goal was to detect treatment effects on a whole genome basis which was achieved by comparative whole genome microarray gene expression profiling of atherosclerosis treatment effects.
4.3. Publication “Microarray gene expression profiling reveals antioxidant-like effects of angiotensin II inhibition in atherosclerosis”

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Microarray gene expression profiling reveals antioxidant-like effects of angiotensin II inhibition in atherosclerosis

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Running title: antioxidant effects of atherosclerosis treatment

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4.3.1 Abstract

Reactive oxygen species (ROS) is a significant feature of atherosclerosis but the impact of ROS on atherogenesis is not clear since antioxidants such as vitamin E have little effect on atherosclerosis development in vivo. To investigate the role of ROS in atherosclerosis, we used ApoE-deficient mice, and compared the treatment effect of the antioxidant vitamin E with that of the angiotensin-converting enzyme (ACE) inhibitor, captopril, because angiotensin II is a major source of ROS in the vasculature. Dihydroethidium staining demonstrated that vitamin E and captopril both prevented the atherosclerosis-induced increase in aortic superoxide content. In contrast, seven months of vitamin E treatment retarded the development of atherosclerotic lesions by only 45.8±11.5 % whereas captopril reduced the aortic plaque area by 88.1±7.5 %. To discriminate between vitamin E-sensitive and -insensitive effects of ACE
inhibition, we performed whole genome microarray gene expression profiling. Gene ontology and immunohistology analyses showed that vitamin E and captopril prevented atherosclerosis-related changes of aortic intima and media genes. However, vitamin E did not reduce the expression of probe sets detecting the aortic recruitment of pro-inflammatory immune cells while immune cell-specific genes were normalized by captopril treatment. Moreover, vitamin E did not prevent the atherosclerosis-dependent down-regulation of perivascular nerve-specific genes, which were preserved in captopril-treated aortas. Taken together, our study detected antioxidant vitamin E-like effects of angiotensin II inhibition in atherosclerosis treatment regarding preservation of aortic intima and media genes. Additional vitamin E-insensitive effects targeting atherosclerosis-enhancing aortic immune cell recruitment and perivascular nerve degeneration could account for the stronger anti-atherogenic activity of ACE inhibition compared to vitamin E.

4.3.2 Introduction

Increased generation of reactive oxygen species (ROS) is a prominent feature of atherosclerosis development (200) (493). The vasoactive peptide angiotensin II was identified as a major trigger of ROS generation in the cardiovascular system (494). Vice versa, inhibition of the angiotensin II system reduced the generation of oxidative stress in vitro and in vivo (467) (495). Concomitantly, inhibition of angiotensin II generation or angiotensin II AT1 receptor antagonism/deficiency retarded the development of atherosclerosis in animal models of atherosclerosis and patients with cardiovascular disease (467) (470) (495) (496). From those data it was concluded that angiotensin II-dependent ROS generation contributed to the development of atherosclerosis (495) (497) (498).

On the other hand, the sole inhibition of ROS by antioxidants and/or genetic tools showed varying results in animal models of atherosclerosis (499). And clinical studies did not detect any reliable effect of antioxidants on the treatment or prevention of cardiovascular disease (500). Nevertheless, many studies confirmed that antioxidants had the potential to decrease the generation of ROS in vitro and in vivo (501) (502) (503) (504) (505). In view of those conflicting results between cellular and animal models, and clinical studies, the impact of ROS on the pathogenesis of atherosclerosis is still not clear.

To study the interplay between angiotensin II and ROS during the development of atherosclerosis, we applied hypercholesterolemic ApoE−/− mice, which are prone to atherosclerosis and reproduce many features of atherosclerosis in patients (506) (507). Moreover, increased ROS generation of ApoE−/− mice was confirmed by several studies (467) (508). To inhibit the generation of ROS, we used the antioxidant vitamin E, which is reported to decrease ROS and also the development of atherosclerotic plaques of ApoE−/− mice when fed a normal diet (503). The generation of angiotensin II was suppressed by the angiotensin-converting enzyme (ACE) inhibitor captopril, which has a well-established atherosclerosis-inhibitory activity in animal models and patients (467) (470) (509). Treatment effects of vitamin E and captopril were compared by quantitative assessment of atherosclerotic plaques and whole genome microarray gene expression profiling. With this approach we sought to identify differences between captopril and vitamin E treatment, which could account for the weak anti-atherosclerotic effect of vitamin E in vivo. Our study revealed vitamin E-like effects of ACE inhibition regarding prevention of atherosclerosis-induced alterations of the aortic intima and media whereas aortic recruitment of pro-inflammatory immune cells and neurodegeneration of perivascular nerves were not sensitive to vitamin E treatment.
4.3.3 Materials and Methods

4.3.3.1 Atherosclerosis treatment of ApoE\(^{-/-}\) mice

The study was performed with ApoE\(^{-/-}\) mice on a B6 (C57BL/6J) background similarly as described (510). Mice were kept on a 12 h light/12 h dark regime, had free access to food and water, and were fed a standard rodent chow containing 7 % fat and 0.15 % cholesterol (AIN-93-based diet; without addition of tocopherol acetate). As indicated, ApoE\(^{-/-}\) mice (age 4-6 weeks) were treated for 7 months without or with captopril in drinking water (20 mg/kg; dissolved fresh every day) or tocopherol acetate (vitamin E, supplied in diet, 2000 IU/kg diet). A control group of B6 mice was also included in the study. At an age of 32-34 weeks, all mice were anesthetized with ketamine and xylazine (100/10 mg/kg), perfused intracardially with sterile PBS, aortas were isolated, rapidly dissected on ice and immediately frozen in liquid nitrogen or processed for further use. Atherosclerotic lesion area was quantified of oil red O-stained aortas by quantitative image analysis.

All animal experiments were performed in accordance with NIH guidelines, and reviewed and approved by the local committee on animal care and use (University of Zurich).

4.3.3.2 Whole genome microarray gene expression profiling

Whole genome microarray gene expression profiling was performed essentially as described previously (510). Total RNA was isolated from aortic tissue of four groups of mice: untreated ApoE\(^{-/-}\) mice, captopril-treated ApoE\(^{-/-}\) mice, vitamin E-treated ApoE\(^{-/-}\) mice and B6 mice. The RNA was processed for whole genome microarray gene expression profiling as described (510). Fragmented, biotin-labeled cRNA (15 \(\mu\)g/ gene chip) was hybridized to the gene chip (Affymetrix GeneChip MG430 2.0 Array with more than 45,000 probe sets) in 200 \(\mu\)l of hybridization solution in a Hybridization Oven 640 (Affymetrix) at 45 °C for 16 h. GeneChips were washed and stained using the Affymetrix Fluidics Station 450 according to the instructions of the manufacturer. Microarrays were scanned with the Affymetrix GeneChip Scanner 7G, and signals were processed to a target value of 200 using GCOS (version 1.4, Affymetrix). Gene ontology (GO) analyses of microarray data were performed with GCOS/RMA processed data using GeneSpring GX software (Agilent). Data were compared between groups using the unpaired two-tailed Student’s t-test. Probe sets with significant difference (i. e. \(P \leq 0.01\) if not otherwise stated, \(\leq\text{-2-fold or } \geq+2\text{-fold difference, with call present and/or signal intensity } \geq 100\) between treated ApoE\(^{-/-}\) mice relative to untreated ApoE\(^{-/-}\) mice were used for GO classification. Microarray data are available at the NCBI GEO database, accession numbers GSE19286 and GSE42813.

4.3.3.3 Histology analyses and immunodetection of proteins

For immunohistochemistry analyses, we used aortic cryo-sections prepared from vitamin E-treated, captopril-treated and untreated ApoE\(^{-/-}\) mice, and from B6 control mice. Isolated aortas of different groups were fixed with formalin (10 % in PBS), dehydrated and frozen at -80°C. Frozen aortas were cut by a cryomicrotome (Microm). Aortic cryo-sections (10 \(\mu\)m) taken at intervals of 50 \(\mu\)m were prepared of the ascending aorta between the aortic sinus and aortic arch region, which is a highly susceptible region for atherosclerotic lesion development. Prior to immunohistochemistry analysis, antigen retrieval was performed by incubation in retrieval buffer (4.2 g citric acid/2 L H\(_2\)O\(_2\), 0.05 % Tween-20; pH 6.0) and heating for 30 min in a microwave. After washing with PBS, the sections were incubated in H\(_2\)O\(_2\) solution (3 % in PBS) for 5 min, to inactivate endogenous peroxidases. After washing steps, sections were incubated in blocking buffer (5 % bovine serum albumin, 0.05 % Tween-20 in PBS) for 1 h. Thereafter, sections were
incubated for 1 h at room temperature with the primary antibody (dilution 1:200 in blocking buffer), followed by three washing steps with washing buffer (0.05 % Tween-20 in PBS) for 5 min each to remove unbound antibody. After incubation with the secondary antibody-peroxidase-conjugate against rabbit [F(ab)2-fragments, dilution 1:500 in blocking buffer] followed by washing steps, the detection of bound antibody was performed by an enzyme-substrate reaction using DAB (3,3′-diaminobenzidine tetrahydrochloride) as substrate (DAB Enhanced Liquid Substrate System; Sigma). The following antibodies were used: anti-CCR9 (raised in rabbit against recombinant CCR9); anti-Cd8b (raised in rabbit against a recombinant protein corresponding to amino acids 22-175 of Cd8b); anti-neuropeptide Y (raised in rabbit against recombinant neuropeptide Y); anti-Pln (raised in rabbit against a peptide corresponding to amino acids 2-25 of Pln); anti-SNAP25 (raised in rabbit against recombinant SNAP25); anti-Sprr3 (raised in rabbit against recombinant Sprr3). Aortic superoxide content was determined by dihydroethidium (DHE) staining of aortic cryosections followed by quantitative assessment of superoxide-generated fluorescence similarly as described (511). Quantification of immunohistology data was done on four animals/group, using ten sections (10 μm) per mouse taken at intervals of 50 μm of the ascending aorta between the aortic sinus and aortic arch region, similarly as described (470). Quantitative assessment of aortic proteins by immunoblotting was performed essentially as described (512).

For atherosclerotic lesion quantification, isolated aortas were opened longitudinally, fixed in formalin (10 % in PBS, 21 h) and stained with oil red O. For oil red O staining, a stock solution of oil red O (0.3 g oil red O in 10 ml of 2-propanol) was prepared and filtered (Whatman grade 1 filter paper). The stock solution was freshly diluted 6:4 with H2O followed by sterile filtration (0.2 μm). For oil red O staining, formalin-fixed aortas were rinsed with H2O and incubated with 2-propanol (70 %). The internal lumen of the pinned aortas was stained with the diluted oil red O solution for 20 min, followed by a brief incubation in 2-propanol (70 %) and rinsing with H2O. Oil red O-stained atherosclerotic lesion area was quantified by image analysis with SigmaScan Pro software.

Unpaired, two-tailed Student’s t-test was used to calculate P values. Analysis of variance was performed with Prism (GraphPad). Statistical significance was set at a P value of < 0.05, unless indicated otherwise.

4.3.4 Results

4.3.4.1 Angiotensin-converting enzyme inhibition by captopril or antioxidant treatment with vitamin E retarded the formation of atherosclerotic lesions in ApoE−/− mice

To investigate the antioxidant effect of angiotensin II inhibition on atherosclerosis, we compared the treatment effect of the ACE inhibitor captopril with that of the antioxidant vitamin E. As disease model of atherosclerosis, we used hypercholesterolemic ApoE−/− mice. After treatment for 7 months, aortas were dissected and atherosclerotic lesion area of oil red O-stained aortas was quantified of vitamin E-treated and captopril-treated ApoE−/− mice relative to untreated ApoE−/− mice (Fig. 1A). Aortic lesion quantification showed that vitamin E had retarded the development of atherosclerotic plaques leading to a decrease in atherosclerotic lesion area by 45.8 ± 11.5 % (Fig. 1A, B). In agreement with previous data, angiotensin II inhibition by captopril had largely prevented the development of atherosclerotic plaques of ApoE−/− mice (Fig. 1A), i.e. the atherosclerotic plaque area was reduced by 88.1 ± 7.5 % in captopril-treated mice compared to untreated ApoE−/− mice (Fig. 1B). As a control, captopril had significantly reduced the systolic blood pressure of ApoE−/− mice from 130.8 ± 3.2 mmHg to 114.6 ± 5.0 mmHg whereas vitamin E had no effect on blood pressure (Fig. 1C).
Figure 1 - Angiotensin-converting enzyme inhibition by captopril and antioxidant treatment with vitamin E retarded the formation of atherosclerotic plaques in ApoE−/− mice. (A) Representative oil red O-stained aortas isolated from a 34 week-old untreated B6 control mouse, a 34 week-old untreated ApoE−/− mouse, a vitamin E-treated ApoE−/− mouse (+VitE), and an ACE-inhibitor-treated ApoE−/− mouse (+ACE-I). (B) Atherosclerotic lesion area was quantified by quantitative image analysis of oil red O-stained aortas isolated from 32-34 week-old ApoE−/− mice, vitamin E-treated ApoE−/− mice and ACE-inhibitor-treated ApoE−/− mice (n = 12 mice/group; ***, P<0.0001 vs. ApoE−/−). (C) Systolic blood pressure of different treatment groups of mice (+SD; n=5 mice/group; ***, P<0.0001 vs. B6). (D) Detection of aortic ROS in situ by dihydroethidium (DHE) staining of aortic sections and quantitative assessment of relative fluorescence levels generated by reaction of dihydroethidium with superoxide (+SD; n=5 mice/group; ***, P<0.0006 vs. B6). (E) Representative DHE-stained aortic sections from a 34 week-old untreated B6 control mouse, a 34 week-old untreated ApoE−/− mouse, a vitamin E-treated ApoE−/− mouse (+VitE), and an ACE-inhibitor-treated ApoE−/− mouse (+ACE-I); bar 200 μm.
4.3.4.2 ACE inhibition by captopril and vitamin E treatment prevented the increase in aortic superoxide content of ApoE\(^{-/-}\) mice

We asked whether the aortic ROS production was affected by captopril or vitamin E treatment. The aortic superoxide content was determined \textit{in situ} by dihydroethidium (DHE) staining, which reacts with superoxide to form a fluorescent product, 2-hydroxyethidium (511) (513). Quantitative fluorescence evaluation of DHE-stained aortic sections revealed a significantly increased superoxide content of untreated ApoE\(^{-/-}\) aortas, i.e. superoxide-dependent fluorescence was increased 2.7 ± 0.4-fold compared to B6 control mice (Fig. 1D,E). In contrast, vitamin E and captopril largely prevented the increase in aortic ROS of ApoE\(^{-/-}\) mice, because the fluorescence of DHE-stained aortas from vitamin E- and captopril-treated ApoE\(^{-/-}\) mice was not significantly different from B6 control level (Fig. 1D, E). Thus, vitamin E and captopril both exerted antioxidant-like effects in vivo, in atherosclerosis-prone ApoE\(^{-/-}\) mice.

4.3.4.3 Whole genome microarray gene expression profiling of atherosclerosis treatment with vitamin E and captopril revealed concordantly regulated aortic genes

To investigate gene expression changes induced by the treatment of ApoE\(^{-/-}\) mice with vitamin E and captopril, respectively, we performed whole genome microarray gene expression profiling of aortic tissue isolated from vitamin E-treated and captopril-treated ApoE\(^{-/-}\) mice relative to untreated ApoE\(^{-/-}\) mice with prominent atherosclerotic plaques. As a control, aortic tissue of healthy, non-transgenic B6 mice was also analyzed. Gene expression data showed a uniform quality of the hybridized microarray gene chips from 4 different groups of mice as evidenced by a comparable number of probe sets present (Fig. 2). RNA integrity of all groups was demonstrated by the comparable 3'/5' signal intensity ratios of probe sets detecting housekeeping genes such as GAPDH (Fig. 2).

Data filtering was performed to detect significantly different probe sets between treated and non-treated ApoE\(^{-/-}\) mice. Data filtering showed that vitamin E treatment had significantly altered the signal intensity of 180 probe sets whereas ACE inhibitor treatment with captopril had significantly altered 469 probe sets (Fig. 3). Among significantly different probe sets, more
than 30 % of vitamin E-regulated probe sets, i.e. 58, showed concordant regulation with probe sets affected by ACE inhibitor treatment (Fig. 3, upper panel).

On the other hand, 14 % of captopril-regulated probe sets showed concordant regulation with probe sets regulated by vitamin E treatment (Fig. 3, upper panel). Together these results indicate that a significant proportion of ACE inhibitor-regulated probe sets are sensitive to treatment with the antioxidant vitamin E.

Concordantly regulated genes between vitamin E- and captopril-treated ApoE−/− mice could be of significant relevance for the pathogenesis of atherosclerosis because more than 82% (i.e. 48) of those commonly regulated probe sets were normalized towards B6 control level (Fig. 3, lower panel).

**Figure 3** - Whole genome microarray gene expression profiling of atherosclerosis treatment with vitamin E and captopril revealed concordantly regulated aortic genes. The upper panel presents a Venn diagram illustrating that 58 significantly different probe sets showed concordant regulation between aortas isolated from vitamin E-treated (VitE) and captopril-treated (ACE-I) ApoE−/− mice relative to untreated ApoE−/− mice. Probe sets with significant difference (P≤0.01; ≥2-fold or ≤2-fold difference, signal intensity ≥100 and/or call present) between vitamin E-treated and non-treated ApoE−/− mice (180), and captopril-treated and non-treated ApoE−/− mice (469) were identified and used for further analysis. The lower panel illustrates that 82.8 % (i.e. 48 probe sets) of concordantly regulated probe sets between vitamin E- and captopril-treated ApoE−/− aortas showed normalization towards B6 control level.
Figure 4 - Identification of atherosclerosis-related aortic genes of untreated ApoE⁻/⁻ mice, which were normalized by vitamin E (VitE) and ACE inhibitor (ACE-I) treatment towards B6 control level. The first panel presents a heat map of genes with low expression in untreated ApoE⁻/⁻ aortas, and the second panel presents probe sets with high expression in untreated ApoE⁻/⁻ mice (P≤0.01, and ≥2-fold or ≤2-fold difference).
Among concordantly regulated probe sets, which were normalized towards B6 control level, 26 probe sets had significantly lower signal intensities in untreated ApoE<sup>−/−</sup> aortas (Fig. 4, upper panel) whereas 22 probe sets showed a higher expression in untreated ApoE<sup>−/−</sup> mice (Fig. 4, lower panel). We focused on those 48 probe sets, which were normalized towards B6 control level, to gain insight into mechanisms underlying the antioxidant-sensitive component of atherosclerosis treatment by the ACE inhibitor captopril.

### 4.3.4.4 Vitamin E and ACE inhibitor treatment maintained the integrity of aortic intima genes of atherosclerosis-prone ApoE<sup>−/−</sup> mice

Gene ontology (GO) analysis was performed of concordantly up-regulated probe sets from vitamin E- and captopril-treated ApoE<sup>−/−</sup> aortas. Gene ontology analysis identified that the majority of genes with ≥2-fold higher expression compared to untreated ApoE<sup>−/−</sup> aortas were associated with stratified epithelium (Fig. 5). Moreover, expression of those genes was normalized towards B6 control level (Fig. 5). According to a previous study, genes associated with stratified epithelium are characteristic of the aortic intima and could protect the aortic intima against biomechanical stress (514).

![Microarray gene expression data of concordantly regulated genes with high expression in vitamin E-treated (VitE) and ACE-inhibitor treated (ACE-I) ApoE<sup>−/−</sup> aortas relative to untreated ApoE<sup>−/−</sup> mice (≥2-fold higher signal intensity; P≤0.01) and normalization towards B6 control level. GO analysis classified the majority of those genes with high expression as stratified epithelial genes of the aortic intima, which could preserve the biomechanical barrier function of the aortic intima. Relative gene expression of concordantly regulated genes is presented as –fold of untreated ApoE<sup>−/−</sup> mice.](image)

Immunohistology analysis confirmed the microarray data for small proline-rich protein 3 (Sprr3) as a typical gene, which was more than 3-fold up-regulated by vitamin E (Fig. 6). Sprr3 showed prominent localization in the aortic intima and adjacent media of a vitamin E-treated and ACE inhibitor-treated ApoE<sup>−/−</sup> mouse, respectively, whereas the Sprr3 protein was barely detectable in the aorta of an untreated ApoE<sup>−/−</sup> mouse (Fig. 6). Immunohistology also indicated that aortic Sprr3 was maintained at B6 control level by vitamin E and captopril treatment (Fig. 6). Together these findings are compatible with the notion that vitamin E treatment and ACE
inhibitor treatment protected the aortic intima of atherosclerosis-prone ApoE−/− mice against ROS-mediated damage.

**Figure 6 - Left panels:** Immunohistological detection of Sprr3 with anti-Sprr3 antibodies validated microarray data and showed down-regulation of Sprr3 in the aorta of a 34 week-old untreated ApoE−/− mouse relative to an age-matched B6 mouse (upper panels). The Sprr3 protein was preserved by vitamin E treatment and ACE-inhibitor treatment (lower panels). Nuclei were counterstained with hematoxylin, HE (bar: 40 μm). The **right panel** shows quantitative evaluation of immunohistology data from four mice each (±SD; n=4; a, P=0.0029; b, P=0.0014; c, P=0.0015 vs. ApoE−/−).

**4.3.4.5 Antioxidant and ACE inhibitor treatment preserved the contractile phenotype of aortic media**

We also performed GO analysis of concordantly regulated probe sets with low expression in treated ApoE−/− mice compared to untreated ApoE−/− mice. GO analysis identified aortic muscle-specific genes as the major category of probe sets (12 probe sets), which showed a significantly lower expression in treated ApoE−/− mice relative to untreated ApoE−/− mice (Fig. 7A). Notably, vitamin E and captopril preserved most aortic media-specific genes at B6 control level (Fig. 7A).

Microarray data were confirmed by immunohistology analysis, which demonstrated the significant up-regulation of the muscle-specific phospholamban (Pln) in the ascending aorta of untreated ApoE−/− mice (Fig. 8). In contrast, phospholamban staining was near B6 control level in vitamin E-treated and captopril-treated aortas (Fig. 8). Moreover, immunohistology analysis of phospholamban detected the proliferation of phospholamban-positive smooth muscle cells in the aortic media of the atherosclerotic aorta (Fig. 8). This finding is significant because proliferation of vascular smooth muscle cells is a characteristic feature of atherogenesis marking the transition of the contractile phenotype of aortic smooth muscle cells to the synthetic phenotype (515). Thus, antioxidant-like effects of the ACE inhibitor captopril could prevent the switch from the contractile to the synthetic phenotype of smooth muscle cells within the aortic media.
Figure 8 - Vitamin E and ACE inhibitor treatment preserved the contractile phenotype of the aortic media. Left panels: immunohistological detection of phospholamban (Ptn) with anti-Ptn antibodies validated microarray data and showed up-regulation of Ptn in the ascending aorta of a 34 week-old untreated ApoE−/− mouse relative to an age-matched B6 mouse, a vitamin E-treated ApoE−/− mouse and an ACE-inhibitor-treated ApoE−/− mouse (bar: 100 μm; nuclei were counterstained with hematoxylin, HE). The right panel shows quantitative evaluation of immunohistology data from four mice each (±SD; n=4; a, P=0.0004; b, P=0.0001; c, P=0.0003 vs. ApoE−/−).
4.3.4.6 Pro-inflammatory immune cell recruitment into the atherosclerosis-prone aorta was sensitive to ACE inhibition but insensitive to vitamin E treatment

Angiotensin II AT1 receptor activation exerts a major pro-atherogenic role by stimulating the recruitment of pro-inflammatory immune cells into the atherosclerosis-prone aorta (470) (510) (516) (517). To decipher the impact of ACE inhibitor treatment versus vitamin E treatment on immune cell recruitment, microarray data were filtered according to the following criteria: (i) significantly lower gene expression in captopril-treated compared to untreated ApoE<sup>−/−</sup> mice (P≤0.05 and ≤2-fold down-regulation), (ii) membrane localization, and (iii) immune cell specificity according to GO analysis. Data filtering identified T cell- and macrophage-specific membrane proteins, which were significantly reduced by captopril treatment towards B6 control level whereas markers of atheroprotective B cells were not decreased (510) (Fig. 9). These findings confirm that the recruitment of pro-inflammatory T cells and macrophages into the atherosclerosis-prone aorta of ApoE<sup>−/−</sup> mice is enhanced by angiotensin II and can be reduced by ACE inhibition (510).

In contrast to captopril, antioxidant treatment with vitamin E did not significantly decrease immune-cell-specific markers in the atherosclerosis-prone aorta (Fig. 9). Immunohistology analysis confirmed the microarray data for the pro-atherogenic T cell and macrophage-resident chemokine receptor 9, Ccr9 (Fig. 10). Vitamin E treatment did not prevent the appearance of Ccr9-positive cells in the atherosclerosis-prone aorta of ApoE<sup>−/−</sup> mice whereas the aorta of captopril-treated mice resembled the B6 control and did not show significant Ccr9-positive cells (Fig. 10).
In agreement with inhibition of the aortic recruitment of pro-inflammatory T cells by captopril, ACE inhibitor treatment with captopril prevented the atherosclerosis-related increase in the aortic content of the T cell-specific Cd8b protein of ApoE−/− mice as determined by immunoblotting (Fig. 11). In contrast, vitamin E treatment did not significantly change the amount of Cd8b protein in the atherosclerosis-prone aorta of ApoE−/− mice compared to untreated ApoE−/− mice (Fig. 11). Together these findings present strong evidence that the recruitment of pro-inflammatory immune cells into the aorta of ApoE−/− mice is enhanced by angiotensin II and sensitive to ACE inhibition but insensitive to vitamin E treatment.
4.3.4.7 ACE-inhibition prevented the atherosclerosis-related down-regulation of perivascular nerve-specific genes of ApoE<sup>−/−</sup> mice

Cardiovascular diseases involving atherosclerosis, hypertension or diabetes are reported to cause perivascular nerve deficits, which finally lead to perivascular nerve degeneration (518) (519) (520) (521). In view of those studies, we asked whether hypercholesterolemic ApoE<sup>−/−</sup> mice also develop perivascular nerve degeneration. To identify significantly altered nerve-specific genes, GO analysis was performed searching for genes associated with a neuron-specific component (e.g. dendrite, myelin sheath or neuronal cell body) and/or the process of neuronal system development. That approach identified 30 significantly altered neuron/nerve-specific probe sets, which were significantly down-regulated more than 2-fold in the atherosclerotic aorta of untreated ApoE<sup>−/−</sup> mice relative to B6 controls (Fig. 12A). Thus, the aorta of atherosclerosis-prone ApoE<sup>−/−</sup> mice is characterized by a significant down-regulation of neuron-specific genes, which could reflect the development of perivascular nerve degeneration.

Angiotensin II inhibition is reported to prevent perivascular nerve degeneration in spontaneously hypertensive rats with excessive angiotensin II generation (521). Since hypercholesterolemia also triggers the release of angiotensin II (522), we asked whether captopril treatment prevented the down-regulation of neuron-specific genes in hypercholesterolemic ApoE<sup>−/−</sup> mice. Microarray gene expression profiling showed that ACE-inhibition prevented the down regulation of all atherosclerosis-associated nerve-specific genes (Fig. 12A). In contrast to ACE inhibition, treatment with vitamin E did not substantially affect those nerve-specific genes (Fig. 12A).
Microarray data on the atherosclerosis-related down-regulation of nerve-specific genes were validated by immunoblot detection of the synaptosomal-associated 25 kDa protein (Snap25) as a neuronal synapse-specific protein. Immunoblot detection revealed that the Snap25 protein level was reduced by 56.1% in aortic tissue of untreated ApoE^-/- mice compared to B6 control aortas whereas the aortic content of Snap25 protein of captopril-treated ApoE^-/- mice was preserved at B6 control level (Fig. 12B). In contrast to captopril, vitamin E did not prevent the atherosclerosis-related decrease of the aortic Snap25 protein content (Fig. 12B).
Neuropeptide Y (Npy) is present in all sympathetic nerves innervating the cardiovascular system (523). Npy and other sympathetic nerve-associated genes [e.g. dopamine beta-hydroxylase (Dbh), dopa decarboxylase (Ddc), and norepinephrine transporter (Slc6a2)] were also among those genes, which showed significant down regulation in the atherosclerotic aorta of ApoE\(^{-/-}\) mice, indicating that perivascular nerve degeneration could affect peripheral sympathetic nerves (Fig. 13). To validate microarray data of sympathetic nerve-associated genes, we performed immunohistological detection of Npy. Immunohistology analysis of the neuron-specific Npy confirmed the microarray data and showed that Npy-positive neurons were decreased by 58.1 % in the atherosclerotic ApoE\(^{-/-}\) aorta compared to the B6 control (Fig. 13). Moreover, captopril treatment maintained the presence of Npy-positive neurons in the aortic adventitia whereas the appearance of Npy-positive neurons was significantly reduced in the vitamin E-treated aorta (Fig. 13). Together these findings strongly suggest that atherosclerotic ApoE\(^{-/-}\) mice develop perivascular nerve degeneration, which is sensitive to angiotensin II inhibition but insensitive to vitamin E treatment.

**Figure 13 - Left panels:** Immunohistological detection of neuropeptide Y (Npy) showed down-regulation of Npy-positive neurons in the ascending aorta of an ApoE\(^{-/-}\) mouse relative to the B6 control (upper panels). Vitamin E treatment (VitE) did not substantially prevent the down-regulation of Npy-positive neurons whereas captopril (ACE-I) treatment prevented the decrease in Npy-positive immunoreactivity in the aorta of an ApoE\(^{-/-}\) mouse (bar: 100 \(\mu\)m; nuclei were counterstained with hematoxylin, HE). The **right panel** shows quantitative evaluation of immunohistology data from four mice each (±SD; n=4; a, P=0.0075; b, P=0.0095 vs. ApoE\(^{-/-}\)).
4.3.5. Discussion

Exaggerated generation of reactive oxygen species (ROS) is considered to affect major processes during the pathogenesis of atherosclerosis. However, the full impact of ROS on atherogenesis is not clear because inhibition of ROS generation by genetic or pharmacological tools has only modest effects in animal models or patients with atherosclerosis (499) (500). To further investigate the role of ROS in atherogenesis, we used atherosclerosis-prone hypercholesterolemic ApoE<sup>−/−</sup> mice, and performed treatment with the antioxidant vitamin E. The treatment effect of vitamin E was compared with that of the ACE inhibitor captopril because angiotensin II is an important contributor to ROS in the vasculature and cardiovascular system (494). Moreover, the anti-atherogenic potential of ACE inhibitors is well established in animal models and patients (467) (470) (495) (496). Dihydroethidium staining showed that both treatment regimens normalized the increased superoxide generation of atherosclerosis-prone ApoE<sup>−/−</sup> aortas. Superoxide is the major vascular-damaging ROS triggered by angiotensin II and drives the generation of other ROS such as hydrogen peroxide and peroxynitrite (524) (525) (526) (527). Therefore, comparable superoxide reduction indicates overlapping antioxidant effects of vitamin E and the ACE inhibitor, captopril. While we detected comparable antioxidant effects of vitamin E and captopril, ACE inhibition was more effective in slowing the development of atherosclerotic plaques, i.e. vitamin E treatment for 7 months retarded atherosclerotic lesion development by 45.8±11.5 % whereas the atherosclerotic plaque area of captopril-treated aortas was reduced by 88.1±7.5 %. That observation strongly suggests that ACE inhibition could exert ROS-dependent and ROS-independent anti-atherogenic effects.

To identify atherosclerosis-related pathomechanisms with concordant sensitivity to vitamin E treatment and ACE inhibition, we performed whole genome microarray gene expression profiling of aortic genes. Searching for significantly altered probe sets with concordant regulation between vitamin E and captopril, we found that more than 82 % of those concordantly regulated probe sets were normalized towards B6 control level. Since treatment-related normalization towards B6 control level could indicate a potential involvement in atherosclerosis lesion development, we focused on those probe sets with concordant regulation between vitamin E and captopril, which showed normalization towards B6 control level.

GO analysis of probe sets with significantly higher expression after treatment compared to untreated ApoE<sup>−/−</sup> aortas and immunohistology analysis revealed that vitamin E treatment and ACE inhibition prevented the atherosclerosis-related down-regulation of aortic intima genes of ApoE<sup>−/−</sup> mice. Identified aortic intima genes such as Sprr3 were previously associated with stratified epithelium, and considered to strengthen the aortic intima against biomechanical stress (514). In this respect our findings are complementary to recent observations, which indicated that ROS is involved in the degeneration of the aortic intima by enhancing the development of endothelial dysfunction (524). Underlying mechanisms could involve inactivation of the atheroprotective nitric oxide (NO), reduced NO synthesis and/or eNOS uncoupling (528). Since angiotensin II-induced activation of NADPH oxidases in endothelial cells is an important contributor to the generation of ROS in the vasculature (529) (530), our microarray study strongly suggests that angiotensin II-stimulated ROS generation could exert a substantial role in deteriorating the endothelial layer, although the precise role of identified genes in atherogenesis remains to be determined.

GO analysis of probe sets with significantly lower expression upon treatment compared to untreated ApoE<sup>−/−</sup> aortas detected that vitamin E and ACE inhibition prevented the up-regulation of muscle-specific genes in the atherosclerosis-prone aorta of ApoE<sup>−/−</sup> mice. Immunohistology analysis confirmed this finding and showed that up-regulation of muscle-
specific genes such as phospholamban in the aortic media correlated with the proliferation of smooth muscle cells close to atherosclerotic plaques, a process, which marks the transition of the contractile to the synthetic phenotype of vascular smooth muscle cells. Since the initial discovery by Griendling et al. in 1994 (531), the involvement of ROS-induced angiotensin II generation in proliferation of smooth muscle cells and vascular hypertrophy is supported by numerous in vitro and in vivo studies (494). Our findings complement those studies by showing that vitamin E treatment and ACE inhibition mediated similar effects concerning the protection of the aortic intima and media in atherosclerosis-prone ApoE−/− mice. Taken together, our findings are compatible with the concept that ROS aggravates the pathogenesis of atherosclerosis. However, the sole inhibition of angiotensin II-induced and NADPH-dependent generation of ROS does not seem sufficient to prevent the development of atherosclerosis (500). Therefore, ACE inhibition could exert additional anti-atherogenic activities. Such anti-atherogenic effects of angiotensin II inhibition seem to be largely blood-pressure-independent because lowering of blood pressure without angiotensin II inhibition, e.g. by hydralazine, did not reduce atherosclerotic lesion area of ApoE−/− mice (468).

In search for additional, vitamin E-independent mechanisms, which could contribute to the anti-atherogenic potential of ACE inhibitors, we focused on the activity of angiotensin II to promote the aortic recruitment of pro-inflammatory immune cells (470) (516) (517). The microarray study and immune techniques demonstrated and confirmed that angiotensin II inhibition reduced the aortic recruitment of pro-inflammatory immune cells. While the atherosclerosis-promoting activity of angiotensin II-stimulated aortic immune cell recruitment is firmly established (470) (510) (516) (517), our current microarray study revealed that the aortic recruitment of pro-inflammatory cells was apparently insensitive to vitamin E treatment because vitamin E did not prevent the atherosclerosis-related increase of immune-cell specific gene expression in the aorta. Since pro-inflammatory immune cells exert a substantial role in atherogenesis (200) (532), the latter observation could - at least partially - explain major differences in the anti-atherogenic activity of ACE inhibition relative to vitamin E treatment observed in animal models and patients.

In addition to inflammatory immune cell migration, which is a well-established factor in atherogenesis, whole genome microarray gene expression profiling revealed another, largely unrecognized atherosclerosis-related process in ApoE−/− mice, i.e. the degeneration of perivascular nerves of the aortic adventitia. Notably, the atherosclerosis-prone aorta of ApoE−/− mice was characterized by a significant down-regulation of multiple nerve-specific genes. Down-regulation of neuronal genes could affect sympathetic nerves, as reflected by down-regulation of DOPA decarboxylase, dopamine beta-hydroxylase, the norepinephrine transporter Slc6a2, and the sympathetic nerve-associated neuropeptide Y. Immunohistology confirmed gene expression data and showed that neuropeptide Y-positive neurons were significantly decreased in the aortic adventitia of ApoE−/− mice.

Down regulation of perivascular nerve gene expression was largely prevented by ACE inhibition with captopril. In contrast, down-regulation of neuronal genes was not substantially affected by vitamin E treatment. These findings could indicate that perivascular nerve degeneration in atherosclerosis could be promoted by excessive angiotensin II generation. Since perivascular nerve degeneration of spontaneously hypertensive rats was also prevented by angiotensin II inhibition (521), angiotensin II and/or high blood pressure seem to exert a common nerve-degenerating effect in the cardiovascular system. In agreement with that notion, perivascular nerve deficits and/or degeneration were detected in different cardiovascular diseases with exaggerated angiotensin II generation such as hypertension, diabetes and atherosclerosis (518) (519) (520) (521). At present, the pathophysiological role of perivascular
nerve degeneration is not fully understood, and additional research in this area is urgently needed. However, some previous studies indicated that the atherosclerosis-related degeneration of perivascular nerves, notably a local decrease in sympathetic nervous activity, could render the vasculature more susceptible to atherosclerosis by increasing the accumulation of collagen and lipids in the vessel wall (533), and reducing the adaptive-trophic influence of sympathetic nerves on vascular structure (534) (535).

Taken together our study showed that the anti-atherogenic potential of ACE inhibition could be partially attributed to antioxidant vitamin E-like effects in the aortic intima and media. However, major atherosclerosis-related activities of angiotensin II inhibition were not sensitive to vitamin E treatment such as prevention of aortic recruitment of pro-inflammatory immune cells and degeneration of perivascular nerves. Those major differences between ACE inhibition and vitamin E treatment could account for the documented anti-atherogenic activity of ACE inhibitors in patients compared to the weak effect of vitamin E.

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5. Inhibition of G Protein-Coupled Receptor Kinase 2 Prevents the Dysfunctional Cardiac Substrate Metabolism in Fatty Acid Synthase-Transgenic Mice

5.1. Introduction to Heart Failure (HF)

5.1.1 Epidemiological landmarks of Heart Failure

Lifetime risk and incidence

Heart failure is a common disease, especially in the industrialized countries. Individuals who are 40 years or older have a 20% lifetime risk to develop heart failure in the US (536). The incidence of HF increases with age. Between 65 and 69 years of age the incidence measures 20 per 1000 person-years and rises to 80 per 1000 person-years in individuals of 85 years of age and over. More than 650,000 individuals get diagnosed with HF each year in the US, and there are more than 23-26 million patients diagnosed with heart failure worldwide (537).

Prevalence

Over 5 million patients with HF live in the US alone. Prevalence of HF is on the rise, mainly due to increasing life-time and improved therapy. Prevalence is highest in persons above 80 years of age (538) (539).

![Image](image.png)

Figure 5.1.1. Prevalence of heart failure by sex and age in the US (from: Bui et al., Nat. Rev. Cardiol. 8, 30-41, 2011)

Mortality

Heart failure is one the diseases with the highest mortality. Half of the patients diagnosed with heart failure die within five years after the initial diagnosis. The ARIC study showed that admission to hospital is an alarming event for heart failure patients which indicates increased risk of death (540). One of ten patients died within the first 30 days after admission to hospital,
22% died within one year after admission. The 5-year mortality rate after admission to hospital was 42%.

**Hospitalizations**

Over 1 million patients are admitted to hospital for treatment of heart failure every year in the US. Due to the severity of this disease and many comorbidities 25% of patients are re-admitted within one month.

### 5.1.2 Definition and classification of Heart Failure

This chapter is based on definitions provided by the ACC/AHA guidelines (541).

Heart failure is a clinical syndrome caused by malfunction of the heart. Causes for this malfunction are an impaired ventricular filling of the heart or a reduced ejection of blood. Heart failure has many possible causes. Due to the typical symptoms of heart failure, irrespective of its causes, it qualifies itself as a disease. Heart failure must not be confused with other CVD, which can result in heart failure, e.g. cardiomyopathy or left ventricular dysfunction.

#### 5.1.2.1 Definition of ejection fraction

The ejection fraction (EF) is the ratio of the amount of blood in the heart to the amount of blood pumped out by the heart. It can be measured by echocardiography (ultrasound examination of the heart) and other cardiac imaging techniques. Generally, the left ventricular EF is considered to be the most important indicator of heart function. After measuring right ventricular EF the global EF of both ventricles can be calculated. In most cases, only the EF of the left ventricle is measured in clinical practice. A left ventricular ejection fraction above 50% is considered normal.

Until some years ago symptomatic heart failure was firmly associated with a reduced ejection fraction. Study results showed that reduced ejection fraction is not a prerequisite for symptoms of heart failure and resulted in the classification into heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF).

#### 5.1.2.2 Heart Failure with reduced Ejection Fraction (HFrEF)

HFrEF is defined as the combination of classical symptoms of heart failure and a reduced ejection fraction of 40% or below. The left ventricular systolic function is reduced, in most cases accompanied with an associated diastolic dysfunction. The major cause for heart failure with reduced ejection fraction are coronary artery disease (CAD/CHD) and myocardial infarction (MI). Several other common causes exist, e.g. dilative cardiomyopathy, hypertensive heart disease, infectious myocarditis, arrhythmia, damaged heart valves or chronic metabolic diseases like diabetes.

Most randomized controlled trials (RCTs) investigating heart failure have only included patients suffering from this type of heart failure. Consequently, the efficacy of most medications used to treat heart failure has only been demonstrated for heart failure with reduced ejection fraction.

#### 5.1.2.3 Heart failure with preserved ejection fraction (HFpEF)

The diagnostic criteria for HFpEF are an ejection fraction of over 40% and clinical symptoms of heart failure (542). The ejection fraction is either normal or only slightly reduced in these
patients, i.e. the ejection fraction is largely preserved. Patients with a left ventricular ejection fraction (LVEF) between 40% and 50% constitute an intermediate group located between patients with HFrEF and patients with symptoms of heart failure and an ideal EF above 50%. The left ventricular diastolic function is disturbed, this can either be detected by Doppler measurement during echocardiography or by cardiac catheterization. Risk factors for developing HFpEF are the same as for other CVD, namely hypertension, obesity, CAD, diabetes, hyperlipidaemia and atrial fibrillation. These diseases and conditions have a high prevalence among patients with HFpEF or HFrEF. The most important cause of HFpEF for which studies have given evidence is hypertension. The prevalence of hypertension among individuals with HFpEF is high and varies between 60% and 89%, depending on the study.

Some patients with HFpEF have recovered from HFrEF, constituting a distinct group of patients, which is in need of further characterization. Until now the pharmacological therapy of HFpEF relies on therapy schemes for treatment of HFrEF.

5.1.2.4 Asymptomatic Left Ventricular Dysfunction (LVD)

Individuals with asymptomatic LV dysfunction have an elevated risk of 10% to develop heart failure symptoms within one year. The annual risk to be hospitalized or to die amounts to 8% for persons with asymptomatic LV dysfunction, their risk is much higher than for the normal population. In most cases, asymptomatic LV dysfunction is accompanied by diastolic dysfunction. The prevalence of LV diastolic dysfunction is often underestimated. Mild LV diastolic dysfunction could be detected in 21% and moderate or severe diastolic dysfunction could be measured in 7% of an English study population which only included participants above 45 years of age. Individuals with LV diastolic dysfunction had an increased risk to develop symptomatic heart failure and to die.

5.1.3 Classification of Heart Failure

Two systems are currently in use for classifying HF according to its severity. While the NYHA scale focuses on the severity of symptoms caused by heart failure, the AHA classification takes abnormalities of heart structure and risk factors into account (541).

5.1.3.1 The New York Heart Association (NYHA) functional classification

The New York Heart Association's classification was first published in 1928 and has been updated several times since then. It focuses on impaired functionality and reduced exercise capacity, which are caused by heart failure. The classification of HF according to the NYHA guidelines is an independent predictor of mortality. The symptomatic status of heart failure is determined by subjective assessment by the patient and is largely defined by the severity of symptoms. In contrast to other classification systems, patients can frequently change NYHA classes, according to the severity of their symptoms which can be influenced by pharmacological therapy or co-morbidities. The NYHA functional classification (Stages I-IV) can be complemented by levels of objective evidence for heart failure or cardiovascular disease and its symptoms collected by healthcare professionals (Levels A-D).

Patient symptoms
- NYHA I
  The patient has no limitation of normal physical activity. Ordinary activities do not cause symptoms of heart failure like dyspnoea or fatigue. No medical interventions aiming at heart failure or its symptoms are recommended at this stage.
- **NYHA II**
  Ordinary physical activity triggers symptoms of heart failure, but patient has no symptoms of heart failure at rest.

- **NYHA III**
  Less than ordinary physical activity causes symptoms of heart failure.

- **NYHA IV**
  All physical activities result in symptoms of heart failure. Symptoms of heart failure are even present at rest.

### 5.1.3.2 The American Heart Association (AHA) classification

The AHA classification of heart failure is focused on development and progression of heart failure. In contrast to the NYHA classification, risk factors are included as indicators for heart failure in a pre-diagnostic stage. Abnormalities in organ and tissue structure resulting in structural heart disease are emphasized. Regression from a more severe stage to a less severe stage of heart failure as defined by the AHA classification is not possible. Progression of heart failure to a more severe category is associated with a reduction of the 5-year survival rate. According to these guidelines therapeutic intervention is necessary even for patients with heart failure of stage A, which is the presence of risk factors for heart failure.

- **Stage A**
  Individuals with high risk for developing heart failure. So far no structural heart disease. No symptoms of heart failure have occurred.

- **Stage B**
  Structural heart disease, but no symptoms of heart failure.

- **Stage C**
  Structural heart disease combined with symptoms of heart failure

- **Stage D**
  Refractory heart failure which does not sufficiently respond to treatment.

The aims of therapeutic intervention change according to the stage of heart failure. In heart failure of stage A, the modification of risk factors is the single aim, possibly preventing structural changes of the heart and progression of heart failure. In stage B the treatment of structural heart disease and the prevention of progression into symptomatic heart disease is essential. In case of symptomatic heart failure (stages C/D) the primary aim is the reduction of symptoms and mortality.

### 5.1.4 Selected Pathomechanisms of Heart Failure

#### 5.1.4.1 Diseases and comorbidities associated with the development of Heart Failure

All diseases, which can affect the heart’s cellular integrity, its metabolism or its blood supply are potential risk factors for developing heart failure. Consequently, all risk factors for CVD in general must be classified as risk factors for heart failure. Early identification of risk factors and appropriate reduction or therapy of risk factors can postpone heart failure (18) (443) (541) (543).
Hypertension

Hypertension is one of the most frequent causes of heart failure in the US. Women and men with hypertension have a markedly increased risk of developing heart failure when compared to “normotensive men and women”. Increased systolic or diastolic blood pressure are individual risk factors for heart failure, although systolic hypertension is associated with higher risk (544) (545). Higher blood pressure levels and longer “duration of hypertension” are associated with a higher incidence of heart failure. Successful treatment of hypertension can lower the risk for developing heart failure decisively. The reduction of systolic and diastolic blood pressure to normal levels results in a risk reduction of approximately 50% (546) (547) (548) (549) (550). The high prevalence of hypertension of above 25% in the US and the increasing incidence of hypertension with age stress the importance of anti-hypertensive therapy in an ageing population (551).

Diabetes mellitus

Diabetes mellitus has several deleterious effects on the heart. By promoting atherosclerosis and coronary artery disease the heart’s supply with oxygen is reduced. Simultaneously, diabetes can evoke changes in cardiomyocyte metabolism. Obesity and insulin resistance are two risk factors for developing diabetes, but also risk factors for developing heart failure. Clinically manifest diabetes is a serious risk factor for developing heart failure, even in persons without structural pre-existing hear disease (552) (553) (554).

Metabolic Syndrome

The symptoms of metabolic syndrome are diverse. Its symptoms are combinations of metabolic conditions and diseases which can promote each other and are on their own risk factors for CVD. Their combination potentiates their dangerous effects on the cardiovascular system, including the heart. Conditions participating in formation of the metabolic syndrome are abdominal obesity, hypertriglyceridemia, low HDL-C, hypertension, fasting hyperglycemia. If an individual is affected by three of these conditions, the diagnostic criteria for the metabolic syndrome are fulfilled. The prevalence is increasing, in the US over 20% of all persons 20 years of age or older have the metabolic syndrome. Its prevalence increases with age, around 40% of persons 40 years of age or older are affected (555). Treatment of hypertension, dyslipidemia and diabetes mellitus reduce the risk for developing heart failure (556).

Atherosclerotic Disease

Most patients with atherosclerotic diseases are likely to develop heart failure because risk factors promoting atherosclerosis also affect the heart and its blood supply. The effective control of risk factors for CVD according to the guidelines is essential in patients with atherosclerotic disease (557).

5.1.4.2 Cardiac metabolism and metabolic dysfunction

Obesity and diabetes are major risk factors for CVD. Concerning the heart, diabetes can cause diabetic cardiomyopathy (558). This syndrome includes cardiac hypertrophy resulting in reduced cardiac function. Heart failure is the most frequent cause of death in diabetics with type 2 diabetes. Several metabolic changes occur in the pathogenesis of diabetic cardiomyopathy, including insulin resistance which is frequently found in diabetics. The energy metabolism of cardiomyocytes is disturbed, glucose utilization is reduced, and lipid oxidation is increased. As a consequence, triglycerides accumulate in the cardiomyocytes. These metabolic changes increase oxidative stress and mitochondrial dysfunction, eventually leading
to apoptosis. In some animal models impaired cardiac glucose metabolism results in increased production of pro-inflammatory cytokines. The local release of cytokines, e.g. IL-6, MCP-1 and TNF-a is increased in heart failure and cardiac hypertrophy (559). These pro-inflammatory cytokines lead to the activation of NF-kappa B which is involved in several diseases of the heart. Considerable “crosstalk” between disturbed cardiac metabolism and local low-grade inflammatory processes takes place (560). NF-kappa B, PPAR, AMPK and sirtuin-1 are some of the biomolecules involved in the regulatory processes caused by metabolic dysfunction. Structural and functional consequences are concentric left-ventricular hypertrophy, dilated cardiomyopathy and extra-cellular cardiac fibrosis which are seen in the “diabetic heart”, resulting in reduced cardiac output (561) (562) (563) (564).

5.1.4.2.1 Metabolic regulation in the normal heart

The heart is in need of constant supply with energy which is stored in adenosine triphosphate (ATP). The production of ATP is accomplished by the mitochondria. Because of their large demand for energy, each cardiomyocyte is equipped with multiple mitochondria, which produce ATP by oxidative phosphorylation. Under physiological conditions, cardiomyocytes rely on lipids, glucose and lactate for energy production in varying extent (565) (566).

5.1.4.2.1.1 Cardiac lipid metabolism

The major source of cardiac energy are lipids (565). About 70% of cardiac energy production is derived from the oxidation of lipids (Figure 5.1.4.2.1).

![Figure 5.1.4.2.1 Overview of major pathways of cardiac metabolism (from Doenst et al., Circ. Res. 113, 709-724, 2013)](image)

Lipids either reach the cardiomyocyte as free fatty acids (FFA) or fatty acid (FA) esters. Lipids can enter the cardiomyocyte by several ways. Fatty acid translocase, CD36 (FAT/CD36) and long-chain fatty acid transport protein 1 (FATP1) mediate the uptake of lipids, although free
fatty acids can pass the cell membrane by passive diffusion. Fatty acid-binding protein (FABP) is responsible for the intracellular transport of long-chain fatty acids. After entering the cell, long-chain fatty acids are transformed into acyl-CoA molecules by the enzyme acyl-CoA synthetase within the cytoplasm. The acyl-CoAs are then transferred to the mitochondria, which they enter by the action of carnitine palmitoyl transferase 1 (CPT-1). This enzyme is located on the outer mitochondrial membrane and “transfers long-chain fatty acid from acyl-CoA to carnitine,” which results in the generation of acetyl-carnitine. “This is the rate-controlling step in the mitochondrial fatty acid oxidation pathway.” Then acetyl-carnitine passes the intermembrane space and is transported into the mitochondrial matrix by a carnitine-acylcarnitine translocase. After its arrival, acyl-carnitine is transformed into acyl-CoA again by carnitine palmitoyl transferase 2 (CPT-2), which sits on the inner mitochondrial membrane. Acyl-CoA diffuses inside the mitochondrial matrix and is oxidized by beta-oxidation, resulting in the generation of acetyl-CoA. Acetyl-CoA then enters the tricarboxylic acid cycle (TCA cycle). Acyl-CoA molecules derived from short-chain FA can diffuse from the cytosol into the mitochondrial matrix without using the “carnitine shuttle” (565) (567).

Long-chain fatty acid esters cannot cross the mitochondrial membranes on their own and must be transported by the “carnitine shuttle”. Free fatty acids with long chains are normally bound to albumin in the blood, while free fatty acids with short chains can be found unbound in the blood. Fatty acids with very long chains are first “shortened” to octanoyl-CoA in cardiomyocyte peroxisomes by oxidation before being transferred to the mitochondria.

5.1.4.2.1.2 Cardiac glucose metabolism

About 20% of the resting heart’s energy is derived from glucose utilization (565) (Figure 5.1.4.2.1). Glucose is water soluble and is dissolved in the blood serum. It arrives at the heart in dissolved, unbound state. Uptake of glucose molecules into the cardiomyocyte is facilitated by glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) (568). Although GLUT1 can only be found in small amounts in cardiomyocytes, some of them stay integrated in the sarcolemmal membrane and keep up a basal activity allowing the spontaneous influx of glucose. The majority of glucose intake is achieved by GLUT4 which can be found in much larger quantity than GLUT1 in cardiomyocytes. Most of them are in dormant state resting in intracellular vesicles until activated by insulin signaling. Upon uptake into the cardiomyocyte glucose is phosphorylated by hexokinase in the cytosol. Glucose-6-phosphate can then enter glycolysis, resulting in the production of pyruvate. Alternatively, glucose-6-phosphate can be transformed into glycogen which is stored within the cardiomyocytes (565).

Pyruvate derived from glycolysis is transported into the mitochondria by a pyruvate carrier, which is yet unidentified. Pyruvate is then transformed into Acetyl-CoA by the pyruvate-dehydrogenase complex (PDC) by oxidative decarboxylation. Acetyl-CoA then enters the TCA cycle within the mitochondrial matrix. FADH2 and NADH are generated by beta oxidation and TCA cycle, which are electron donators for electron transport chain which ATP synthesis by oxidative phosphorylation.

Insulin signaling

The insulin signaling cascade has great influence on cardiac energy metabolism and regulates the balance between utilizing glucose and lipids as energy substrates (569). Assuming physiological conditions, the insulin receptor is activated upon binding its ligand insulin. Its tyrosine kinase activity results in phosphorylation and activation of insulin receptor substrate-1 (IRS-1) which has a prominent position in maintaining insulin signaling and glucose
metabolism in cardiomyocytes. Activated IRS-1 is capable to stimulate the PI3K pathway and the MAPK pathway.

The PI3K pathway

The activation of the phosphoinositiode 3-kinase (PI3K) by tyrosine-phosphorylated IRS-1 results in the stimulation of a kinase cascade through generation of phosphoinositides which are phosphorylated on the 3-position by PI3K, mostly phosphatidylinositol-3,4,5 trisphosphate (570) (571). This kinase cascade results in triggering the serine/threonine protein kinase B (PKB)/Akt signaling pathway (572). PKB/Akt promotes the translocation of GLUT4 to the cell membrane, which results in increased glucose uptake by the cardiomyocyte, increasing the amount of substrate for glycolysis (573) (574). At the same time PI3K facilitates the translocation of FAT32/CD36 to the cardiomyocyte membrane. The uptake of fatty acids into the cardiomyocyte is promoted but most of them are “stored in intracellular lipid pools” and not metabolized under the influence of insulin and its effectors (575) (576) (577) (578).

Insulin effects result in a shift of substrate utilization in the cardiomyocytes, pushing the proportion of ATP won by glucose utilization up to 70% after supply with carbohydrates. In times of low supply with carbohydrates, the Randle cycle (syn. glucose-fatty acid cycle) prevents the increased metabolism of glucose for energy production (579). Further effects of insulin are increased glucose uptake and intensified utilization of glucose. This shift increases cardiac efficiency and reduces myocardial oxygen consumption (580).

Impaired insulin signaling in the diabetic heart

In diabetes, insulin sensitivity is reduced, the binding of insulin to its receptor and the subsequent effector cascade are diminished. Consequently, basal glucose uptake by the cardiomyocytes is reduced and fewer GLUT4 channels are translocated into the cardiomyocyte membrane. The basal glucose uptake and glycolysis are also reduced. The rate of glucose as substrate for ATP production is negatively affected (580).

Modulators of insulin signaling

Two modulators of insulin signaling are tyrosine phosphatase 1B (PTP1B), which reduces the effects of insulin, and Sirtuin1 (SIRT1), which increases insulin sensitivity and stimulates the effects of insulin (581). PTP1B is a negative regulator of insulin signaling. It is a tyrosine phosphatase and is capable to dephosphorylate phosphotyrosine residues of the insulin receptor, resulting in impaired function of the activated insulin receptor. The receptor’s signaling function is disturbed, the effects of insulin reduced. Sirtuin1 is an indirect positive regulator of insulin signaling and improves insulin sensitivity. It is a nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylase and catalyses the deacetylation of lysine-residues, a process coupled with NAD hydrolysis. The results are a de-acetylated protein and nicotinamide, which is a negative regulator of SIRT1. Increased NAD/NADH ratio and AMP-acitvated protein kinase (AMPK) activity increase the activity of SIRT1 (582). The action of SIRT1 represses the transcription of PTP1B, which is a negative regulator of insulin signaling. In insulin-resistant states the expression of SIRT1 is downregulated. Additionally, NAD levels and AMPK activity are decreased, resulting in reduced activity of already present SIRT1. In the state of insulin-resistance the effects of SIRT1 activation are stronger. SIRT1 action can be stimulated by resveratrol, which itself is a powerful antioxidant. “Sirtuin is the link between caloric restriction” and cellular response to it.” It is also involved in the processes of aging, inflammatory processes and metabolism (583) (584). SIRT1 deacetylates and thereby activates the PGC-1-alpha/ERR-alpha complex, which is an important regulator of metabolism.
Furthermore, SIRT1 can reduce the activity of p53 by deacetylation and has mono-ADP-ribosyltransferase activity.

**AMP-activated protein kinase (AMPK)**

AMPK is another key regulator of cardiac energy metabolism (585) (586). It is upregulated in times of increased demand of ATP, which is the case in heart failure. ATP depletion will finally result in phosphorylation and activation of AMPK, which is achieved by upstream kinases, like liver-kinase B1 (LKB1). AMPK is a hetero-trimeric serin/threonine kinase, consisting of a catalytic alpha-subunit and two regulatory subunits beta and gamma. AMPK plays an important role in cardiac energy homeostasis. It is activity is reduced by insulin signaling, which is an indicator for supply with glucose. In general, actions of the AMPK favor ATP-producing metabolic pathways while they reduce the intensity of ATP-consuming pathways. The level of AMPK activity rises as a reaction towards an increasing AMP/ATP ratio, which shows increased consumption of ATP and high demand for it (568) (587) (588).

Acetyl CoA-carboxylase (ACC) is inhibited by phosphorylation by AMPK, resulting in decreased levels of malonyl-CoA. This compound is an inhibitor of CPT-1, which catalyses the rate-limiting reaction of the “carnitine-shuttle”. In response to the falling levels of malonyl-CoA, the activity of CPT-1 is increased, and more fatty acids are transported from the cytosol into the mitochondrial matrix. There, the fatty acids are the substrate for the process of beta oxidation, which produces acetyl-CoA and reducing equivalents. AMPK regulates the expression of genes involved in beta oxidation and activates available PGC-1a, which is an important indirect stimulator of beta-oxidation (589) (590). AMPK can increase the expression of PGC-1a by increasing the binding of transcription factors to the promoter regions of the PGC-1a gene, leading to increased amounts of mRNA coding for PGC-1a. SIRT1 is also activated by AMPK, it also enhances the function of PGC-1a. Increased AMPK activity does not only stimulate lipid utilization but also enhances glucose utilization for increasing ATP production. This is achieved by elevated expression of GLUT4 and its facilitated translocation into the cell membrane.

In sum, AMPK activates both metabolic pathways, leading to balanced increase of lipid and glucose substrate metabolism. Its activity is modulated by the influence of insulin, which itself suppresses AMPK activity while bolstering glucose metabolism. Metformin is an activator of AMPK and showed to be beneficial in experimental models of heart failure (591).

**5.1.4.2.1.3 The PPAR/ERR/PGC-1 axis**

The main regulators of cardiac energy metabolism on transcription level are the peroxisome proliferator-activated receptors (PPAR), which are found in the nucleus. These transcription factors influence the activity of certain metabolic pathways (592) (593). Three isoforms exist, and the action of each has characteristic effects on the cardiomyocyte metabolism. The ligands of all PPARs are long-chain fatty acids or eicosanoids and some PPAR share the same ligands, but with different binding affinity.

After ligand binding, each PPAR must heterodimerize with the 9-cis retinoic acid receptor (RXR), which is another nuclear receptor, to be activated. After assembly of these heterodimers co-activators with histone-acetylase activity or co-activators mediating the link between PPAR and target gene, are recruited. Subsequently, the activated PPAR-RXR heterodimers bind to PPAR-response elements (PPRE), which are specific nucleotide sequences located in the promoter region of target genes. Now the PPAR gains its function as a transcription factor. The co-activators’ ability to acetylate histones makes the target DNA more accessible for the RNA polymerases. The transcription of PPAR target genes is enhanced upon binding of PPAR-RXR
heterodimers and their co-activators to the PPRE. If no ligands are present, PPAR-RXR heterodimers firmly bind to co-repressor proteins, preventing transcriptional activity. The co-repressors are released again after ligand binding.

**Isoforms of PPAR**

**PPAR-alpha**

This isoform can be found in tissues with high capability to oxidize fatty acids. After activation, the transcription of genes coding for proteins supporting fatty acid metabolism is increased. Consequently, fatty acid uptake, intracellular fatty acid transport and mitochondrial beta oxidation are stimulated by activation of PPAR-alpha. Myocardial glucose metabolism is reduced, the expression of proteins involved in glucose transport and glycolysis is decreased (594). As a consequence, increased expression of PPAR-alpha in experimental heart failure improved myocardial function and cardiac energy metabolism (595).

**PPAR-beta/delta**

This isoform is expressed in most tissues, but is especially abundant in tissues with intense metabolic activity, e.g. skeletal muscle and cardiac muscle. PPAR-beta/delta increases the transcription of genes necessary for glucose metabolism (596). The rate of energy produced by utilizing glucose as a substrate increases. Due to increased myocardial glucose metabolism, oxidative metabolism is increased. More glycogen is gradually removed by glycogenolysis, and less glycogen is stored within the cardiomyocyte. Additionally, PPAR-beta/delta reduced PDC and AMPK expression in mouse models, possibly due to increased and more efficient generation of NADPH (597).

**PPAR-gamma**

In the healthy adult heart, PPAR-gamma is only expressed in small quantity and plays a minor role in comparison to the large amounts of PPAR-alpha and PPAR-beta/delta – as long as PPAR-gamma is present and functions regularly. Knockout of PPARG is deleterious for the heart and results in cardiac hypertrophy and heart failure (598). Transcription and translation of proteins involved in “fatty acid uptake and fatty acid oxidation are reduced”. Myocardial fatty acid oxidation is diminished, and the hearts contractility is reduced. On the other hand, activation of PPAR-gamma or overexpression in the heart is also detrimental and leads to cardiac hypertrophy, dysfunction and lipid overload (598) (599).

**PPARG-coactivator-1 alpha (PGC-1a)-regulator of fatty acid use and beta oxidation**

One of the most prominent co-activators of PPARG which is abundant in cardiac tissue is PGC-1a (600). This co-activator has multiple central functions within the cardiomyocyte. In times of high energy demand its expression is increased. The increased energy demand can be short-term (e.g. fasting) or sustained (e.g. diabetes). Strikingly, its expression is reduced in several diseases of the heart which go hand in hand with abnormally changed energy utilization, including cardiac hypertrophy, heart failure and ischemic heart disease. The best studied role of PGC-1a is being a co-activator of PPARG, but it does also activate other transcription factors in the cardiomyocyte, e.g. NRF-1 (Nuclear Respiratory Factors 1) and ERR-alpha (estrogen-related receptor alpha). It possibly activates PPAR-alpha and PPAR-beta/delta in weaker extent, too. PGC-1a is a vital part of regulatory pathways controlling metabolism and is involved in the regulation of mitochondrial biogenesis, fatty acid beta-oxidation and the electron transport chain. Concerning the heart’s metabolism in organ development and in conditions of stress PGC-1a is a crucial element (601). In the perinatal heart the increased
expression of PGC-1α is responsible for changing the phenotype of cardiac energy substrate utilization from glucose to fatty acid utilization and boosts “mitochondrial biogenesis”. Although its effects primarily promote fatty acid use for energy production, PGC-1α also participates in the regulation of glucose oxidative metabolism, but to a much smaller extent. It can be seen as a metabolic-inducible co-factor.

Myocardial overexpression of PGC-1α in transgenic animal models activates the heart’s energy metabolism, favouring the utilization of fatty acids (602). Expression of proteins involved in the uptake and oxidation of fatty acids is increased, the expression of enzymes and proteins participating in the TCA, the electron transport chain and oxidative phosphorylation is upregulated. This results in a dysregulated mitochondrial metabolism, dilated cardiomyopathy and heart failure. The knockout of PGC-1α also results in “cardiac dysfunction” and symptoms of heart failure. In this case, the utilization of fatty acids for energy metabolism is impaired and the mitochondrial energy transduction and subsequent ATP production are reduced (603).

**PDK-4, a regulator of glucose oxidation**

The enzyme catalyzing the rate-limiting reaction of glucose oxidation is the PDC (pyruvate dehydrogenase complex). One of its most important regulators is PDK4 (pyruvate dehydrogenase kinase-4), which inactivates PDC by phosphorylation and thereby reduces glucose-oxidation. Concomitantly PDK4 promotes lipid oxidation. In diabetes and obesity PDK4 expression is increased, paving the way for insulin resistance by inhibiting the substrate use of glucose for energy metabolism. Consequently, glucose oxidation is decreased and fatty acid oxidation is increased in models of myocardium-specific overexpression of PDK4 in transgenic mice (604). The lack of “metabolic flexibility” concerning substrate use is disadvantageous for the heart, its susceptibility for developing “cardiac fibrosis and cardiomyopathy” is increased. The expression of PDK-4 is regulated by many transcription factors. Some transcription factors which promote PDK-4 and lipid metabolism are co-activated by PGC-1α, these include PPARs, ERRs and FOXO1. Other transcription factors which can balance these effects are E2F1, HNF4, LXR and RXR which are not dependent on PGC-1α (600).

**ERR a,b,c**

The estrogen related receptors (ERR) are nuclear receptors, but this is not the only functional similarity they share with the PPARs (600). Actually, they are central regulators of energy metabolism and are transcription factors promoting “fatty acid oxidation and oxidative phosphorylation”, in part by supporting the transcription of PPAR-alpha. (605) PGC-1α is an important co-activator of ERRs, interacting directly with them. The expression of PGC-1α is in return stimulated by ERR-alpha. No real ligand has so far been identified for ERR-alpha, it is still considered an orphan receptor.

**PI3K-pathway**

The phosphoisoitide 3-kinase (PI3K) pathway is highly active in the heart, independent of its metabolic or functional state. Upon its activation, PI3K phosphorylates phosphoinositides (PI) on the 3-position (606). Several other downstream kinases are recruited and activated by PI3K, including the pivotal PKB/Akt. In concert with PDK-1 and monomeric G proteins the generation of many downstream effectors is induced. Two of these targets, glycogen synthase kinase-3b (GSK-3b) and “mammalian target of rapamycin” (mTor) promote glucose metabolism.
5.1.4.2.2. Metabolic dysfunction in heart failure and diabetic cardiomyopathy

Diabetes is an important risk factor for many severe cardiovascular diseases and heart disease in general which are the most frequent causes of death in diabetics (580). The long-term effects of diabetes include a disturbed cardiac metabolism which can result in the apoptosis of cardiomyocytes and eventually heart failure (560). Incidence of cardiomyopathy is much higher in individuals with diabetes, even after adjustment for other risk factors of cardiovascular disease in frame of the metabolic syndrome (e.g. hypertension, hypercholesterolemia) or secondary diseases like microvascular disease. Although diabetic cardiomyopathy is the second-most frequent cause of heart failure, it has only recently been acknowledged as a distinct disease. It has often been referred to as “idiopathic dilative cardiomyopathy” in the past. One of the first large studies to highlight the increased risk of cardiomyopathy and heart failure in diabetics was the Framingham Heart Study (FHS) (607).

The diagnosis of diabetic cardiomyopathy and resulting heart failure is not much different from the diagnosis of other types of heart failure (558). The decisive diagnostic criterion for diabetic cardiomyopathy is the impaired function of the left ventricle in the diabetic patient, which is not caused by coronary artery disease or hypertension. Usually, the first symptom caused by diabetic cardiomyopathy is diastolic dysfunction, which can often only be detected by echocardiography. In later stages of the disease systolic dysfunction occurs which reduces ventricular function and can reduce ejection fraction. Most patients will only feel symptoms in this more advanced stage of the disease. In advanced diabetic cardiomyopathy, successful therapy is limited to prevention of disease progression and to optimization of the heart’s work. Only the early diagnosis and sufficient treatment of diabetes or even prediabetes can prevent the development of diabetic cardiomyopathy until now. Echocardiographic examinations showed a highly increased prevalence of diastolic dysfunction in patients with diabetes type 2, up to 30% of patients showed this first symptom of heart failure.

Over the last two decades, cardiac metabolic pathways have further been elucidated, making the development of pharmacological agents improving the derailed energy metabolism in diabetic cardiomyopathy of all stages a primary aim of research (569) (608) (609).

The most widely used animal models of diabetes type 1 are streptozocin-induced diabetic models, in which the pancreatic beta cells, which produce insulin, are destroyed by streptozocin. Mice with genetic deletion of leptin (ob/ob mice) or genetic deletion of the leptin receptor (db/db mice) are models of obesity and insulin resistance, resembling diabetes mellitus type 2. The “Zucker fatty rat” is an alternative animal model of diabetes type 2, these animals also develop obesity and insulin resistance (610). Strikingly, animals of all diabetes models mentioned develop cardiac dysfunction on the long run, making them suitable for investigation of diabetic cardiomyopathy. These animal models confirm the pathologic link between diabetes, cardiomyopathy and heart failure (611).

5.1.4.2.2.1 Metabolic changes in the diabetic heart

The reason for many disturbances in diabetic cardiomyopathy and the dysfunction of cardiomyocytes is a derailed metabolism. Therefore, diabetic cardiomyopathy can be considered as “a metabolic disease of the heart” (580). In the resting, healthy heart about 70% of adenosine triphosphate molecules (ATP), which are considered as energy equivalents, are generated from the utilization of fatty acids, while about 30% of energy is generated from utilization of glucose. The utilization of glucose for energy production is increased by physical activity or adrenaline secretion, quickly increasing the heart’s energy generation. The redox equivalents resulting from glycolysis and beta oxidation are used in the process of oxidative
phosphorylation to generate ATP. Mitochondrial oxidative phosphorylation accounts for 95% of total ATP and energy production. The generation of ATP is especially important in the cardiac tissue. The human heart has the largest energy and ATP consumption per gram of tissue of the whole body, the human heart can use up to 6 kg of ATP per day. In order to function correctly, the cardiomyocytes must have metabolic flexibility which allows them to respond to altered requirements and varying supply with fatty acids and glucose (580).

Under physiological conditions, substrate utilization of fatty acids and glucose is regulated by the “Randle cycle” which involves reciprocal regulation through the metabolites of beta-oxidation and glycolysis (579). In diabetes, the balance of supplied energy substrates is disturbed, resulting in an unbalanced Randle cycle with emphasis on promotion of fatty acid beta oxidation (580).

Alterations of metabolic flexibility with a shift to the utilization of more fatty acids for energy production is one of the earliest changes in the diabetic heart and are detectable prior to ventricular dysfunction (612) (613). In the later stages of the disease, the heart relies more or less completely on the metabolism of fatty acids for energy supply. This metabolic switch to complete reliance on fatty acids must be interpreted as an initially protective mechanism, which is harmful on the long run. The heart loses its metabolic flexibility, the loss of glucose as an energy source is deleterious for the diabetic heart. This metabolic shift takes place in the hearts of patients with diabetes type 1 and diabetes type 2. Their hearts showed increased levels of fatty acid metabolism and reduced glucose metabolism in metabolic PET studies (614).

Figure 5.1.4.2.2.2 Overview of changes in the cardiomyocyte’s energy metabolism in heart failure associated with diabetes. Activity of certain pathways or generation of several metabolites is either increased or decreased (white arrows). (from Fukushima et al., Biochim Biophys Acta. 1861(10):1525-34, 2016) (612)
5.1.4.2.2 Molecular mechanisms of metabolic shift in diabetic cardiomyopathy

The increased uptake of fatty acids results in increased expression of genes responsible for lipid metabolism, shifting energy metabolism further to fatty acids (Figure 5.1.4.2.2). Secondly, the increased uptake of fatty acids leads to intracellular accumulation of lipids, increasing generation of potentially toxic lipid intermediates like ceramide and diacylglycerol (DAG). Several animal models of type 1 and type 2 diabetes recapitulate the intra-myocardial lipid accumulation in the diabetic heart, and show an increased rate of fatty oxidation while glucose utilization is reduced. More animal models simulating or stimulating a metabolic shift towards fatty acid utilization have been investigated. Mice with cardiac-specific deletion of the glucose transporter GLUT4 show reduced uptake of glucose and focus on fatty acids for ATP generation, ultimately resulting in cardiac dysfunction resembling diabetic cardiomyopathy in human patients (615). In line with this result, overexpression of GLUT4 in db/db mice caused stronger glucose uptake and protected the heart from dysfunction (616). Upregulation of cellular lipid absorption by overexpression of human lipoprotein lipase in cardiomyocytes resulted in increased uptake of fatty acids originating from circulating VLDL particles, causing lipid accumulation and dilated cardiomyopathy (617).

5.1.4.2.2.1 Disturbed cardiac fatty acid metabolism in diabetes

The uptake of fatty acids into the cardiomyocyte is less strictly regulated than the uptake of glucose. Most fats and fatty acids are taken up by the fatty acid transporters CD36, FATP and fatty acid-binding protein (FABP) while passive diffusion plays a minor role. The amount of fatty acid uptake is partially determined by the concentration of fatty acids in the blood running through the arteries supplying the heart. They arrive at the heart either as singular fatty acids bound to albumin or in the form of triacylglyceride-rich lipoprotein particles (612).

In diabetes, lipolysis is increased, resulting in increased levels of fatty acids and lipoprotein particles in the blood. Consequently, increased levels of fatty acids are taken up by the cardiomyocytes. The increase of intracellular fatty acid concentration has multiple deleterious effects on the cardiac metabolism.

5.1.4.2.2.2 Activation of PPAR-alpha and its target genes

The increased availability of fatty acids finally results in metabolic reprogramming, which is mainly mediated by PPAR-alpha, an important transcription factor of proteins responsible for upregulating fatty acid metabolism (580). Ligand binding assays identified fatty acids as important ligands and activators of PPAR-alpha. Upon its activation, fatty acid uptake and beta oxidation are further increased. At the same time, PPAR-alpha is the transcription factor of proteins which inhibit glycolysis and reduce the utilization of glucose as a source of energy, e.g. by inhibiting pyruvate dehydrogenase kinase 4 (PDK4). The metabolic shift caused by PPAR-alpha activation further generates more by-products derived from beta oxidation, e.g. citrate, which can inhibit enzymes responsible for glucose uptake (GLUT4) and regulation of glycolysis (PFK). The expression of PPAR-alpha itself is also increased in animal models of diabetic cardiomyopathy.

The effects of PPAR-alpha could be confirmed in an animal model with cardiac-specific overexpression of PPAR-alpha, which resulted in a phenotype resembling diabetic cardiomyopathy (618). Cardiac palmitate uptake and metabolism were increased in these mice while glucose utilization was reduced. Histology revealed accumulation of lipids within
cardiomyocytes and showed increased density of lipid droplets while functional imaging detected diastolic dysfunction in murine hearts overexpressing PPAR-alpha.

The transcriptional targets of PPAR-alpha include several enzymes responsible for the uptake of fatty acids into the cardiomyocyte, including CD36 and the fatty acid-binding protein (FABP). Several animal models showed that CD36 is an important factor in the development of metabolic shift and diabetic cardiomyopathy. Fatty acid metabolism could be reduced and glucose utilization for energy production could be increased by the knockout of CD36 in mice. In contrast, mice with CD36 knockout overexpressing PPAR-alpha showed less intracellular triacylglyceride content and had partially restored glucose oxidation. These animals had improved cardiac function in comparison to mice with PPAR-alpha overexpression and intact CD36 production (578). The protein responsible for the transfer of fatty acids into the mitochondrion, carnitine palmitoyltransferase-1 (CPT-1), is also positively regulated by PPAR-alpha. Etomoxir, an inhibitor of CPT-1, stimulated “cardiac glucose utilization” (580).

Another important transcription target of PPAR-alpha is PDK4, which inhibits pyruvate dehydrogenase, the enzyme responsible for the rate-limiting reaction of glycolysis. Rat models of diabetes have increased PDK4 protein levels and show enhanced activity of this inhibitor of glycolysis. Increased levels of PDK4 could also be detected in murine hearts overexpressing PPAR-alpha, confirming previous reports. By this mechanism PPAR-alpha decreases glucose utilization and further strengthens “metabolic remodeling” within the diabetic heart, keeping its energy metabolism in an inflexible condition with strong focus on fatty acid utilization for energy production (580).

The overexpression of peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1a), which is an important co-activator of PPAR-alpha and strengthens its transcriptional activity, results in upregulation of genes involved in fatty acid uptake and fatty acid metabolism and finally in the development of dilated cardiomyopathy and heart failure, resembling diabetic cardiomyopathy. PGC-1a is also an important co-factor of the nuclear estrogen related-receptors (ERR), which are transcription factors regulating expression of proteins involved in cellular metabolism. ERRα is a transcription factor stimulating the expression of genes coding for proteins involved in fatty acid oxidation and oxidative phosphorylation, PGC-1a is one of its most important activators. Because PGC-1a is also a co-activator of nuclear respiratory factor 1 (NRF1), basal PGC-1a activity is important for mitochondrial health and functioning. Nevertheless, PGC-1a has indispensable other functions, as PGC-1a knockout mice develop heart failure (602).

5.1.4.2.2.2.3 Less efficient generation of energy equivalents (ATP) by beta oxidation

The effect of increased reliance on fatty acids for energy metabolism is disadvantageous for the cardiomyocyte considering the efficiency of substrate metabolism. Generating energy from fatty acids by beta oxidation is less efficient than the generation of energy from glucose by glycolysis. The heart relying solely on utilization of fatty acids for generating energy works inefficiently. The mitochondrial process of beta oxidation requires more oxygen than the process of glycolysis, while it produces relatively fewer reduction equivalents necessary for the generation of ATP by oxidative phosphorylation in the respiratory chain. Consequently, the ratio between expended oxygen molecules and generated ATP molecules is much larger if fatty acids are utilized for energy generation by beta-oxidation (566).

Analysis in the heart showed that the metabolism of 1 g palmitate consumes 46 atoms of oxygen while 105 molecules of ATP are generated. In contrast, the oxidation of 1 g glucose requires only 12 atoms of oxygen and produces 31 molecules of ATP. The generation of one molecule
ATP through metabolism of fatty acids requires about 0.3 oxygen molecules more than the generation of one molecule ATP by metabolism of glucose (619).

Accordingly, cardiac oxygen consumption is increased by over 30% in hearts of diabetic ob/ob mice when compared to hearts of control animals (580). At the same time, contractile force generated by the hearts of diabetic ob/ob mice was equal or even reduced in comparison to the hearts of non-transgenic mice. The increase of oxygen consumption can also be triggered by infusion of fatty acids into control animals or healthy humans. Dogs which were infused a mixture of heparin and triacylglyceride had their cardiac oxygen use increased by 25%, but cardiac power was unchanged (620) (621). After the increase of circulating fatty acids by infusion of a heparin triglyceride mixture non-transgenic healthy pigs showed a reduction of cardiac contraction force (622). Isolated mouse hearts without any mutation also reacted with increased oxygen extraction towards perfusion with free fatty acids (623).

Taken together, the reduced metabolic efficiency of fatty acid beta-oxidation finally results in increased oxygen consumption and less generated ATP which is the heart’s fuel for pumping.

5.1.4.2.2.4 Increased insulin resistance and inhibition of glycolysis by fatty acids

In diabetes, the secretion of insulin or the insulin sensitivity are reduced. Due to lack of intracellular glucose, this can lead to an increased activity of the sympathetic nervous system and increased lipolysis, resulting in higher concentration of fatty acids in the circulation. This is the first step in the cardio-metabolic shift towards preferred utilization of fatty acids as a source of energy. The increased uptake of fatty acids by the cardiomyocytes results in an elevated intracellular concentration of fatty acids and their derivatives. By activating intracellular serine kinases, e.g. protein kinase C, insulin receptor substrate 1 (IRS1) is phosphorylated next to the binding site of the phosphoinositide-3 kinase, making it impossible for the phosphoinositide-3 kinase to bind to IRS1. As a result, the activation of IRS1 by phosphoinositide-3 kinase is reduced and insulin signaling is impaired, causing insulin resistance which further intensifies the metabolic shift away from glycolysis to beta-oxidation of fatty acids. The inhibition of IRS1 and insulin signaling by free fatty acids could be confirmed in cell culture experiments, animal models and human studies. Insulin resistance can be promoted by activity of the sympathetic nerve system. This is one mechanism how the over-activated sympathetic nervous system causes insulin resistance (569) (580).

The enhanced fatty acid metabolism, which is accused of promoting insulin resistance, can also inhibit glycolysis directly by increased production of acetyl-CoA and NADH in the process of fatty acid oxidation. The latter two can stimulate the PDK4, which is an important inhibitor of the PDH complex that catalyzes the mitochondrial oxidation of pyruvate, the product of glycolysis (624) (625). Acyl-CoA can also inhibit phosphofructokinase-1 allosterically, which is the first enzyme of glycolysis (626). This mechanism further aggravates the metabolic dysfunction of the diabetic heart.

5.1.4.2.2.5 Increased lipotoxicity

The intracellular accumulation of lipids is regularly found in diabetic cardiomyopathy and includes the concentration of ceramides and diacylglycerol within the cardiomyocyte (627). Ceramide and diacylglycerol interfere with several intracellular signaling pathways and can influence energy metabolism by decreasing the cardiomyocyte’s insulin sensitivity. Ceramide and diacylglycerol achieve this by reducing the tyrosine phosphorylation of insulin receptor substrate, resulting in the desensitization to insulin and thereby potentially reducing glucose uptake and metabolism. Additionally, they can promote apoptosis by triggering “release of
cytochrome c from the mitochondria”. In transgenic mouse models developing lipotoxic cardiomyopathy, inhibition of ceramide synthesis prevented progression of cardiac dysfunction and stabilized cardiac function and energy metabolism. The inhibition of ceramide synthesis by myriocin was able to lower cardiac ceramide levels and increased cardiac insulin sensitivity in mice on a high-fat diet (628).

Diacylglycerol is generated in the process of lipolysis, which is increased in obesity and diabetes. Its ability to activate protein kinase C (PKC) can disrupt cardiac insulin signaling and reduce glucose uptake by the cardiomyocyte. Mice receiving a high-fat diet showed reduced glucose oxidation in response to insulin stimulation. This effect was accompanied by accumulation of diacylglycerol in cardiomyocytes. Increased degradation of diacylglycerol by DGAT1 overexpression protected mice from cardiac dysfunction in a mouse model of lipotoxicity, although more tri-acylglycerides were produced (629). The role of triglyceride accumulation in diabetic cardiomyopathy is still unclear, but its effect is not as dangerous as that of ceramides and DAG. The data concerning lipotoxicity within the human diabetic heart is still limited, but data collected from other organs and animal models of diabetic cardiomyopathy suggest an important role in the development of cardiac metabolic dysfunction (630) (631) (632).

Ceramides and diacylglycerol are not the only lipids which exhibit toxic effects. Derivatives of long chain acyl-CoA are capable of inhibiting the transport of generated ATP from the mitochondrion to the cytosol by inhibiting the cardiomyocyte’s adenine nucleotide translocator (633) (634). This could delay delivery of ATP and energy to the sarcomers, which need constant supply with ATP to contract. The increased uptake of fatty acids can also result in the recurrent intracellular conversion of triacylglycerides into acyl derivatives and vice versa, a process called “cycling” of lipid intermediates (635). This may be a protective mechanism, but it consumes lots of energy. Cycling of lipid derivatives consumed 30% of cellular energy in isolated non-contracting cardiomyocytes in fatty acid rich media.

5.1.4.2.2.2.6 Mitochondrial dysfunction

Enzymes involved in the thermogenesis of hibernating animals and newborn children are the uncoupling proteins, which are mainly expressed in brown fat tissue. In brown adipocytes, this uncoupling is supposed to generate heat to prevent decrease of body temperature (636) (637) This is achieved by integration of UCP into the mitochondrial membrane, constituting a “pore” or shortcut between the mitochondrial inter-membrane space and the matrix of the mitochondrion (638). This pore allows the flux of protons from the inter-membrane space into the matrix, following their gradient. Energy is released as heat, leading to thermogenesis. The transfer of protons from the inter-membrane space to the matrix is separated from the generation of ATP, leading to mitochondrial uncoupling and thermogenesis.

The expression of uncoupling proteins is not limited to brown adipocytes, and can be induced in other cells, including cardiomyocytes. One metabolic factor is the increased availability of fatty acids, which increases UCP2 and UCP3 expression even in non-diabetic animal models and human studies. In diabetes, the fatty acid metabolism of cardiomyocytes is upregulated, which results in increased expression and activation of uncoupling proteins in the heart. Consistent with increased fatty acid availability, rodent models of diabetes showed upregulation of cardiac UCP expression. The expression of some uncoupling proteins is promoted by PPAR-alpha, which itself is activated by fatty acids. Pharmacological activation of PPAR-alpha in control animals also resulted in increased expression of UCP2 and UCP3 (639). Knockout of PPAR-alpha reduced the expression of UCP significantly in the murine heart (640). Due to mitochondrial uncoupling within the cardiomyocytes less ATP is generated
which makes the heart’s metabolism less efficient. Slightly enhanced activity of uncoupling proteins may attenuate generation of ROS by “mild uncoupling” (641) (642). In contrast, a strong increase of uncoupling in the mitochondria, e.g. by palmitate-CoA or UCPS, can result in increased generation of ROS in the mitochondria, aggravating mitochondrial dysfunction (643). Mitochondrial uncoupling further reduces the metabolic efficiency of the heart relying on beta-oxidation. As a consequence, less ATP is produced while oxygen consumption is increased (644).

5.1.4.2.2.7 Increased oxidative stress

The investigation of cardiac mitochondria of diabetic cardiomyocytes revealed mitochondrial damage and reduced mitochondrial respiration, possibly due to elevated oxidative stress. Increased concentration of free fatty acids within the cardiomyocytes can trigger generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by non-mitochondrial sources and mitochondrial sources, of which the latter are especially important. These reactive molecules can modify proteins or nucleotides, resulting in cellular dysfunction or even apoptosis. Due to the increased consumption of oxygen, more reactive oxygen species are generated in the mitochondria of diabetic hearts, including superoxide, resulting in mitochondrial and cellular damage which aggravates cardiomyopathy (645). The cardiac mitochondria of animal models overexpressing an antioxidant such as metallothionein or catalase showed reduced oxidative stress while the heart had improved contractility in comparison to animals not overexpressing the antioxidant.

5.1.4.3 The role of GRK2 in Heart Failure

The G protein-coupled receptor kinase (GRK2) is an enzyme involved in regulating several important signaling pathways in the cardiomyocyte, which influence cardiac metabolism, functioning and viability. Although GRK2 is expressed by many different cell types, it is the GRK with the highest expression level in the heart. The heart is an organ with intense power output, which must be regulated to prevent damage or exhaustion.

Function of GRK2

GRK2 binds to seven-transmembrane spanning-receptors (7TMRs) associated with heterotrimeric G proteins. Therefore, these receptors also called G protein coupled receptors (GPCRs). The GRK2 is a kinase, which was initially identified to regulate the activity of the beta-adrenoceptor by phosphorylation (646). Activated beta-adrenoceptors with bound agonist are a target of GRK2, which phosphorylates the activated beta-adrenoceptor. After phosphorylation, beta-arrestin 1/2 is capable to attach to the beta-adrenoceptor by binding to the phosphorylated cytoplasmatic tail domain of the GPCR (647). The binding of beta-arrestin 1/2 to the beta-adrenoceptor results in desensitization of the receptor and can finally lead to the receptor’s endocytosis, which is clathrin-mediated. Desensitized receptors are inactive and do not respond to binding of their ligand adrenalin while endocytosed receptors leave the membrane and can either be resensitized and brought back to the membrane or are degraded by lysosomes (648). Importantly, beta-arrestins are signaling molecules with many different functions, which can scaffold other proteins, e.g. c-Jun aminoterminal kinase (JNK) and mitogen activated protein kinases (MAPK) including ERK, to constitute multiprotein complexes. GRK2 can also inactivate other GPCRs by phosphorylation. Analogous to GRK2, beta-arrestins are also capable to interact with other receptors (649).
Structure of GRK2

GRK2 consists of three different domains, each of it having its own function (650). It has a central catalytic region, acting as a serine/threonine kinase, which allows specific binding to GPCRs. Attached to the central kinase domain is the RGS homology (RH) domain, which allows regulation of G protein signaling by binding activated Gq/11 subunits, promoting their inactivation. The catalytic domain and the RH domain are flanked by the alpha-helix on the amino-terminal side and by the pleckstrin homology (PH) domain on the carboxyl-terminal side. The amino-terminal alpha-helix domain structurally supports the interaction of the GRK2 with its GPCR while the PH domain interacts with the beta-gamma subunit of the heterotrimeric G-protein which is bound to the membrane. Localization next to the membrane and interaction with membrane phospholipids is made possible by this property of the PH domain (650).

Cellular localization of GRK2

GRK2 is mostly found in the cytosol and to a lesser extent on and in mitochondria. Its expression and protein level increases if ischemic or oxidative stress occurs.

5.1.4.3.1 GRK2 mediates exaggerated beta-adrenoceptor desensitization in Heart Failure

GRK2 is the major desensitizing kinase in the heart. The actions of GRK2 in the heart can either be protective or detrimental, depending on the duration and intensity of its activity (651). Its physiological task is to protect the heart from stress in times of intensive physical activity or to shield the heart from further damage after cardiac injury, e.g myocardial infarction. In these cases, the sympathetic nervous system is overactive, and elevated catecholamine levels result, which increase signaling of the beta-adrenoceptors. Cardiac inotropy increases, but in parallel, energy expenditure and O2 consumption also increase and can promote ischemia and oxidative stress. The negative effects on the heart can be reduced by inactivation of beta-adrenoceptors, which is achieved by GRK2 (Figure 5.1.4.3.1).

![Figure 5.1.4.3.1 GRK2-mediated desensitization of beta-adrenoceptors in the heart.](image-url)
In periods of increased demand, GRK2 can protect the heart against increased energy expenditure, ischemia, increased heart rate and ischemic stress by deactivation of the beta-adrenoceptors, making them unresponsive towards catecholamines. This is achieved by phosphorylation of the activated beta-adrenoceptor, which is a G-protein coupled receptor (GPCR). GRK2 is translocated to the activated GPCR by binding to the beta-gamma subunit of the G-protein associated with the beta-adrenoceptor. As a consequence of GRK2-mediated receptor phosphorylation, the adaptor protein, beta-arrestin binds to the phosphorylated receptor, which interrupts the interaction of the G-protein alpha subunit with the receptor.

By desensitization-mediated inhibition of beta-adrenoceptors, GRK2 reduces the heart’s contractility and inotropy. Chronic over-activity of GRK2 results in permanent deactivation and desensitization of the cardiomyocytes’ beta adrenoceptors, reducing the hearts inotropic reserve for responding to increased demand signaled by catecholamines. The heart’s contractility and pumping function are diminished, two symptoms frequently found in heart failure. As a result of the heart’s inability to increase its output, the sympathetic nervous system is even activated further, releasing more catecholamines via the baroreceptor reflex, which stimulate GRK2 activity. A vicious circle is established, which prevents the inactivation of GRK2 resulting in chronic depression of heart contractility. In agreement with this, GRK2 levels are significantly increased in the hearts of patients with heart failure and in the hearts of animal heart failure models (651).

5.1.4.3.2 Non-canonical functions of GRK2 with relevance to heart failure

The most studied cellular function of GRK2 is the desensitization of beta-adrenoceptors and other GPCRs. For that reason, this function is also called the “canonical” or “classical” function of GRK2. Other functions, which have only been recently discovered, are regulation of cardiac metabolism and cardiomyocyte apoptosis by GRK2. These functions involve phosphorylation and regulation of non-GPCR substrates by GRK2. As a consequence, these functions are termed “non-canonical” or “novel” functions of GRK2 (652).

Many studies have been conducted to analyze the effects of GRK2 inhibition on cardiac function. Genetic animal models of GRK2 down-regulation and pharmacological inhibition of GRK2 achieved resensitization of the beta-adrenoceptors on the long term, and normalized the number of beta-adrenoceptors, improving the heart’s function. The inhibition of GRK2 was able to reverse symptoms of heart failure induced by myocardial ischemia in several animal studies and prevented the development of heart failure in several heart failure models. These results support the thesis that the vicious circle of continuous stimulation of the sympathetic nervous system and beta-receptor deactivation by GRK2 can be broken by GRK2 inhibition.

5.1.4.3.2.1 GRK2 and cardiac metabolism

GRK2 has several functions, which can influence the substrate use for the generation of energy in the cardiomyocyte, mainly through its ability to modulate insulin resistance. Levels of GRK2 are increased in conditions and diseases with insulin resistance, which is a hallmark of diabetes, obesity and ageing (653). The insulin receptor substrate 1 (IRS-1) is an adapter protein necessary for the assembly of the “insulin receptor signaling complex”, which is responsible for downstream signaling of the insulin receptor. GRK2 can reduce the function of IRS-1 by phosphorylating its Ser307 residue, which prevents formation of the “insulin receptor signaling complex” or can result in the dissociation of a pre-existing “insulin receptor signaling complex” (654). In cardiac myocytes overexpressing GRK2, the expression and activity of important “downstream effectors” of the insulin receptor, which include Akt and GLUT4, are reduced. While GRK2 can directly bind to and inhibit Akt, it also reduces its activation by inhibiting
IRS, which is capable of stimulating Akt activity via PI3K and PIP3 signaling. By inhibiting this PI3K/Akt pathway, GLUT4 translocation to the membrane is also reduced by GRK2 (654).

A functional inhibitor used as a GRK2 inhibitor in many cell culture and in vivo models is the BARK-C-terminus, which is the C-terminus of GRK2 (655). It inhibits GRK2 by scavenging of beta-gamma subunits of heterotrimeric G-proteins, which are required for GRK2 activation and translocation to the GPCR substrate (655). The BARK-C-terminus was used to inhibit the GRK2 activity, which is increased in response to insulin signaling. Long term treatment with insulin also leads to increased GRK2 levels. Overexpression of the BARK-C-terminus resulted in a decreased phosphorylation of IRS-1 after administration of insulin, which is normally a stimulus for GRK2 activity. These results indicate that the interaction of GRK2 with IRS-1 depends on the C-terminus of the GRK2. Glucose uptake improved after expression of the BARK-C-terminus in a model of myocardial ischemia, Akt activity and GLUT4 translocation to the membrane were normalized (654).

Several animal models with reduced expression or inhibited function of GRK2 have been investigated and showed that the inhibition of GRK2 exerts beneficial effects on glucose metabolism (653) (656). The tissue specific knockout of GRK2 in cardiomyocytes resulted in an increased glucose uptake and a significantly reduced phosphorylation of IRS-1, thereby supporting insulin signaling (654). Mice with systemic heterozygous knockout of GRK2 have a more stable glucose metabolism and are more resistant against the development of insulin resistance (653).

Taken together, GRK2 activation leads to an impaired glucose uptake by the heart and a phenotype of insulin resistance. This phenotype is a hallmark of several cardiomyopathies leading to heart failure, e.g. diabetic cardiomyopathy. Vice versa, GRK2 inhibition with the BARK-C-terminus counteracted this cardiometabolic dysfunction under experimental conditions.

Another non-GPCR, which activates GRK2, is the insulin-like growth factor 1 receptor (IGF-1R), which is essential for cell growth. IGF-1 receptor-stimulated signaling results in increased levels of GRK2 in the breast carcinoma cell line, MCF7, by inhibiting mdm2 and ubiquitin ligase, which are responsible for the degradation of GRK2 (657). At the same time, GRK2 is able to desensitize the receptor tyrosine kinase bound to the IGF-1 receptor, resulting in the inhibition of the IGF-1 receptor. These effects of GRK2 could be relevant for the cardiac cell metabolism because IRS-1 also interacts with the IGF-1 receptor.

5.1.4.3.2.2 GRK2 and mitochondrial dysfunction

Several scientific findings suggest that GRK2 is involved in the regulation of mitochondrial metabolism. GRK2 could be detected on the membranes of cardiomyocyte mitochondria and inside of cardiomyocyte mitochondria (658). Only few details about the function of GRK2 in mitochondria are known yet, but mitochondrial GRK2 may modify ATP production. No binding partners of GRK2 in mitochondria have been identified so far. In case of increased GRK2 expression after ischemic injury, the binding of GRK2 to mitochondria is increased. Binding of GRK2 to the mitochondrial membrane may augment the permeability of the mitochondrial permeability transition pore (MPTP) (659) (660). As a consequence, less calcium accumulation inside the mitochondria could be sufficient to open the pores, and thereby the mitochondrion’s calcium tolerance is reduced. Opening of the pores leads to loss of the proton gradient and subsequent calcium release from the mitochondrion. While the loss of the proton gradient can result in decreased ATP synthesis, the released calcium activates
calcium-dependent proteinases. The release of pro-apoptotic cytochrome c from the mitochondria is also increased after association of GRK2 to the mitochondrial membrane (658).

The shuttle for GRK2 transfer to the mitochondria after generation of reactive oxygen species is the chaperone Hsp90. A prerequisite for the binding of GRK2 to Hsp90 is the phosphorylation of GRK2 on residue Ser670, which is conducted by mitogen-activated protein kinases (MAPK). Expression of the beta-adrenergic receptor kinase (BARK) C-terminus was used a GRK2 inhibitor, and resulted in less binding of GRK2 to the mitochondria by blocking Hsp90. This mechanism could contribute to the observation that the BARK C-terminus protects the heart from cardiomyocyte death after ischemic injury. Mutation of the phosphorylation site of the BARK C-terminus results in the loss of this protective function. Expression of the BARK C-terminus can protect the heart from ischemic injury, the reduction of GRK2 adhering to the mitochondria may be one mechanism (658).

Mice with cardiomyocyte-specific knockout of GRK2 showed a reduced cytochrome c release after ischemia/reperfusion injury, which itself was less severe than in control animals. The harmful effects of extensive GRK2 activity on mitochondria may stimulate aberrant metabolism and contractile dysfunction in heart failure while a reduced activity of GRK2 can improve cardiomyocyte survival (658).

5.1.4.3.2.3 GRK2 regulates the cellular redox state through endothelial nitric oxide synthase

Another important mechanism influenced by GRK2 is the regulation of cellular redox reactions, which are involved in the development of heart failure. Studies investigating the effect of GRK2 overexpression in cardiomyocytes showed that activity of the endothelial nitric oxide synthase (eNOS) is reduced. The interaction between GRK2 and eNOS is a direct, physical interaction, which results in the inactivation of eNOS and reduced NO synthesis. The direct binding between GRK2 and eNOS could be detected by co-immunoprecipitation of protein extractions from transgenic murine hearts and cardiomyocytes overexpressing GRK2. Strikingly, ischemia and oxidative stress promoted the interaction between GRK2 and eNOS, which inhibited each other by binding. Upon binding to GRK2, eNOS induces inactivating S-nitrosylation of GRK2 by NO production. The inhibition of GRK2 by S-nitrosylation on Cys340 promotes cardiac contractility by improving beta-adrenoceptor signaling. These findings imply that increased levels of NO can reduce GRK2 activity, leading to reduced beta-receptor desensitization and probably improved cell survival. Mice with a genetically modified GRK2 mutant, which cannot be S-nitrosylated by eNOS at Cys340, showed an increased activity of GRK2 and increased susceptibility to ischemia-reperfusion injury (661).

Inhibition of GRK2 by expression of the BARK-C-terminus increased the activity of Akt and eNOS, resulting in elevated synthesis of NO in hearts impaired by ischemia-reperfusion injury. The increased expression of GRK2 in heart failure suppresses eNOS activity and reduces NO synthesis, which results in a cycle of continuous suppression of eNOS activity and enhanced GRK2 activity (662).

In summary, GRK2 must be considered a key player in the development of cardiac dysfunction and heart failure, being a “nodal link between cellular metabolism, and cardiac contractility”. Multiple experimental data show that GRK2 inhibition could be a valuable therapeutic approach to counteract the pathological cascade triggered by GRK2 overactivity.
The following chapter gives a brief overview of the diagnosis of heart failure as it is recommended in the guidelines of the ESC (543), and the ACC/AHA/HFSA (541).

The diagnosis of heart failure includes taking a detailed history, focusing on risk factors, and conducting a thorough physical examination. Typical symptoms are consequences of impaired heart function and include signs of reduced exercise tolerance and increased fluid retention. Heart failure symptoms can be distinguished into symptoms of left heart failure and symptoms of right heart failure, depending on the ventricle which is most affected. Fatigue, dizziness, abdominal pain can be signs of reduced output of blood by the heart and reflect that the blood supply of organs is reduced. Fluid retention can result in pulmonary edema, which triggers dry coughing and dyspnea. Other frequent signs of heart failure and fluid retention are peripheral edema, e.g. on the lower limbs and ankles. Imaging, most commonly by echocardiography, can visualize the heart and its actions, giving further hints on probable causes of the disease. The size and function of the ventricles can be assessed, allowing quantification of the ejection fraction. In most cases of heart failure, the left ventricle shows functional abnormalities, e.g. systolic or diastolic dysfunction, abnormal left ventricular size or reduced ejection fraction.

5.1.5.1 Blood Biomarkers

BNP/NT-proBNP

These two biomarkers are cleavage products and derive from its precursor peptide proBNP (663). These three peptides are released by cardiomyocytes after myocardial stretch and other trigger factors. All tests detecting BNP or NT-proBNP also measure proBNP. Increased BNP/NT-proBNP levels indicate heart failure and are a valuable tool for diagnosis of heart failure, and for measuring the severity of heart failure in chronic heart failure and acute decompensated heart failure (664). Usually, low values of BNP exclude heart failure and the presence of increased BNP values is closely associated with heart failure. The successful therapy of chronic heart failure is reflected by decreasing values of BNP which also correlate with better clinical outcomes. Especially younger patients seem to benefit from a BNP “guided” therapy, in which the levels of BNP are interpreted as markers indicating success of therapy.

Cardiac Troponin T and Cardiac Troponin I

These two enzymes are released into the bloodstream by injured or dead cardiomyocytes (663) (665). Elevated levels are found in many patients with heart failure, reflecting increased stress cardiomyocytes are exposed to. Patients suffering from heart failure and CHD show higher serum concentrations of troponin than patients with heart failure alone, because ischemia is one of the most important reasons for cardiomyocyte injury. Elevated troponin levels are associated with increased mortality in chronic heart failure and acute decompensated heart failure. Successful therapy of heart failure can decrease the release of troponin from the cardiomyocytes. Decreasing troponin values in patients treated for acute or chronic heart failure are associated with a more favourable prognosis. Onset or aggravation of heart failure can be associated with acute coronary syndrome or myocardial infarction leading to ischemia. All patients showing symptoms of acute heart failure or patients with decompensated chronic heart failure should be checked for increased troponin T values.
Other biomarkers: Soluble ST2 and galectin-3

These are biomarkers for the degree of myocardial fibrosis. “They are not only predictive of hospitalization and death in patients with HF but also additive to natriuretic peptide levels in their prognostic value.” Soluble ST2 and galectin-3 are not routinely measured in patients with heart failure until now (541) (666).

Biomarkers of renal function or injury are important prognostic indicators in patients with heart failure. The heart and the kidney do rely on each other for functioning properly. Several studies have shown that increasing loss of kidney function is a marker for increasing heart failure. The further loss of function in one of these organs will harm the second organ (663) (667).

5.1.5.2 Imaging

Chest x-ray

Making a chest x-ray is recommended for all “patients with suspected, acute or new-onset heart failure”. If the patient is able to stand or sit upright, making an erect or semi-erect chest x-ray reveals more information than a supine chest x-ray. Cardiac enlargement (cardiomegaly) and pulmonary congestion are the two only direct symptoms of heart failure which can be detected by chest x-ray. The enlargement of heart chambers and their function can be roughly estimated. Increased pulmonary venous pressure can result in visible pulmonary vessels, widened pulmonary veins and pulmonary edema. Calcifications of the valves can be seen as “white” spots (x-ray dense) and hint to a valve dysfunction. The chest x-ray has a low sensitivity and specificity but gives an important first impression of the patient’s cardiovascular condition and can show signs of other thoracic diseases or conditions which can cause heart failure symptoms, e.g. pneumonia or fractures.

Echocardiography (transthoracic or transesophageal)

The most frequent imaging techniques used for diagnosing, quantifying and monitoring heart failure is 2-dimensional echocardiography, which is the ultrasound examination of the heart. This technique does not set free ionic radiation and one ultrasound device is sufficient to conduct an advanced examination of the heart’s anatomy and function. Many different morphologic and functional parameters regarding the heart can be measured and quantified. They include left ventricular diameter (LVD) and left ventricular wall thickness. The most important derived measurement concerning heart failure is the left ventricular ejection fraction (LVEF), which is the percentage of blood ejected from the ventricle with each beat. Other important aspects which can be visualized in the echocardiography are regional wall motion and geometry of the heart chambers. The valves should be controlled for normal shape and motion. The functional status of the valves is additionally assessed by Doppler flow measurement. This technique measuring the direction and speed of blood current is integrated in most modern ultrasound devices. Mitral and tricuspid valves often develop insufficiency during the development of heart failure. The possibility to collect hemodynamic data noninvasively is a big advantage of echocardiography.

The first echocardiographic examination and subsequent serial evaluations are usually conducted through the chest wall (transthoracic). To allow better imaging of certain regions of the heart the ultrasound device’s transducer can be placed in the patient’s esophagus (transesophageal echocardiography). In patients showing symptoms of heart failure, echocardiography should be conducted to determine the functional status of the heart and its morphology. In patients presenting without overt symptoms of heart failure, echocardiography
can reveal subclinical heart failure. Repeated echocardiographic examinations are useful to detect changes in cardiac morphology or function in patients showing reduction or aggravation of heart failure symptoms. Changes in the severity of heart failure symptoms are often associated with structural or functional changes of the heart in the echocardiography.

**Cardiovascular magnetic resonance imaging (CMRI)**

In order to image and measure the cardiac morphological and functional parameters in higher resolution than echocardiography, CMRI can reveal details on myocardial perfusion, viability and fibrosis. These parameters are useful to get more clues on the possible etiology of heart failure and to improve prognosis estimation. While CMRI is free from ionizing radiation, it is one of the most expensive cardiac imaging techniques.

**Cardiac computed tomography (CCT)**

Similar to CMRI, this method of cardiac imaging does also depict cardiac anatomic structures in superior detail and allows very exact quantification of cardiac function. Like CMRI, CCT can give further information on the state of the myocardium, but the practical value of this is still unclear. Coronary arteries can be visualized without contrast media, giving important information on their condition and possible obstructions and calcifications. In case of CAD, this can be helpful to detect the etiology of heart failure. The drawback of CCT is the patient’s exposure to high amounts of ionizing radiation.

**5.1.6 Pharmacological therapy of heart failure with reduced ejection fraction (HFrEF)**

For heart failure, a treatment algorithm guides the therapy according to symptoms and severity (Figure 5.1.6). The treatment guidelines give recommendations according to the currently available clinical trials. A Class-I recommendation indicates that there is “evidence and/or general agreement that a given treatment or procedure is beneficial, useful, effective”, and a Class-IIa recommendation indicates that the “weight of evidence/opinion is in favor of usefulness/efficacy”. Thus, Class-I means that the therapy is “recommended”, and Class-IIa indicates that the therapy should be “considered” (543).
Figure 5.1.6 Therapeutic algorithm for the treatment of symptomatic heart failure with reduced ejection fraction (HFrEF), adapted from: Ponikowski et al., Eur. Heart J. 37, 2129-2200, 2016.
5.1.7 New drugs for the treatment of Heart Failure

In this chapter, two new drugs for the treatment of chronic heart failure with reduced ejection fraction are presented. This is of special interest for the cardiology community because ivabradine and the combined angiotensin receptor and neprilysin inhibitor (ARNI) have been the only two drugs to be approved for the treatment of chronic heart failure by the FDA after a time period of more than ten years without new medications since 2002. Ivabradine and ARNI have been approved by the FDA for the treatment of chronic heart failure in 2015. They have only recently been included in the treatment guidelines for heart failure published by the ESC (543), and the 2013 guidelines by ACC/AHA, which were updated in 2017 (541).

Use of Ivabradine (Corlanor®) in heart failure

The cardiomyocytes of the sinoatrial node are specialized cells, capable to generate action potentials, which spread over the whole heart and induce the heart’s contraction. After their depolarization during the action potential, the cardiomyocytes repolarize. In the phase of membrane hyperpolarization, the HCN channels open. The HCN channels permit a steady flux of sodium ions into the cell. This leads to a continuous, spontaneous depolarization of the membrane in the diastole. Finally, the threshold potential is reached and the HCN channel closes while the action potential is triggered.

Ivabradine is a blocker of the HCN sodium channels, which are located on the membrane of the rhythmogenic cardiomyocytes of the sinoatrial node. By binding to the HCN channel, they block the If (funny) current. This results in slower spontaneous depolarization of the sinoatrial myocytes and a reduced heart rate. The decreased heart rate reduces the oxygen consumption by the heart, and allows the heart to work more economically. At the same time, perfusion of coronary arteries is slightly increased by the prolonged diastole.

Figure 5.1.7-1 Formula of ivabradine.

Although ivabradine has been approved as an antianginal drug in 2005, this chapter focuses on the use of ivabradine in the treatment of patients with chronic heart failure with reduced ejection fraction (668).

Frequency control is one of the ways the heart’s energy and oxygen consumption can be reduced in order to delay the development of heart failure. Several earlier studies investigating pathomechanisms of heart failure, showed a positive linear correlation between an increased heart rate and cardiovascular death. In agreement with this, the resting heart rate is a prognostic marker in patients with heart failure with reduced ejection fraction (669). Until a short time ago, only beta blockers have been widely used to reduce the heart rate. They are now challenged by ivabradine, which might replace them for control of heart rate in certain patients. By reducing the heart rate, ivabradine can reduce oxygen consumption, which is supposed to be the main reason for the beneficial effects of ivabradine. Similar effects can be achieved by beta
blockers, but many patients cannot tolerate sufficient doses, e.g., patients with asthma. The exact mechanisms, why ivabradine can improve survival in heart failure have not been completely elucidated yet. The main data supporting the use of ivabradine in heart failure comes from the SHIFT study, which examined the effects of ivabradine on patients with heart failure and reduced ejection fraction below 35% (668). To prevent bradycardia, which is a common side effects of ivabradine, the minimal resting heart rate of patients to be included in the study had to be above 70 bpm. Patients with arrhythmia were not included in the study. One third of patients included in the SHIFT trial had heart failure unrelated to ischemia. The rate of patients receiving heart failure therapy including ACE inhibitors and beta blockers was high, although the majority of patients did not reach target doses of beta blockers. A major result found by the SHIFT study was that ivabradine reduced the rate of hospitalizations due to worsening heart failure by 18% in comparison to placebo (668). Thus, ivabradine seemed to increase the overall quality of life in the treatment group. In contrast to the rate of hospitalizations, the all-cause mortality and the mortality due to all cardiovascular reasons could not be reduced by ivabradine. Only the mortality due to heart failure itself could be slightly reduced by ivabradine in the SHIFT trial. Despite the largely positive data of the SHIFT trial there were also experts who raised concern about the use of ivabradine in view of a second clinical trial investigating the effects of ivabradine on heart failure, the BEAUTIFUL trial. In the BEAUTIFUL trial cardiac outcomes were not improved by ivabradine (670). In contrast to the SHIFT study, the BEAUTIFUL trial only included patients with heart failure caused by coronary artery disease and ischemia. The safety of using ivabradine in patients with heart failure due to coronary artery disease taking beta blockers could be confirmed in the BEAUTIFUL trial. The BEAUTIFUL trial could not confirm the reduction of composite cardiovascular endpoints, including cardiovascular death or admission to hospital for worsening heart failure. In spite of these findings, patients, who had a resting heart rate above 70 bpm where less often admitted to hospital for myocardial infarction or coronary revascularization if treated with ivabradine. Echocardiographic studies conducted in frame of the BEAUTIFUL trial indicate that therapy with ivabradine may reduce left ventricular dysfunction and cardiac remodeling (670).

It is still unclear, why ivabradine lowered mortality in the SHIFT study but not in the BEAUTIFUL study. It has been hypothesized that ivabradine may be better tolerated or may have more beneficial effects in patients suffering from heart failure, which is not caused by ischemia. As a result of both the SHIFT and BEAUTIFUL clinical trials ivabradine has been included in the ESC guidelines and the NICE guidelines for the treatment of heart failure since 2012 and the American Heart Association has adopted ivabradine into their guidelines for the treatment of heart failure since 2016. The guidelines point out that patients must have a physiological sinus rhythm with a resting heart rate of over 70 bpm to be eligible for ivabradine and that beta blocker doses must have been increased to the tolerated maximum before starting ivabradine.

**Combined angiotensin receptor blocker and neprilysin inhibitor (ARNI)**

A new class of drugs used for the therapy of heart failure are drugs containing an angiotensin receptor blocker and a neprilysin inhibitor. Their benefit is a synergistically enhanced therapeutic effect on the pathophysiology of heart failure. So far, the only drug of this class which has been approved is Entresto® which contains the angiotensin receptor blocker valsartan and the neprilysin inhibitor sacubitril. Entresto® has only been approved for the therapy of heart failure with reduced ejection fraction in 2015, and has been included in the guidelines for therapy of heart failure by the ESC (543), and the 2013 guidelines by ACC/AHA/HFSA, which were updated in 2017 (541).
Mechanism of drug action of neprilysin inhibitors and ARNIs

This chapter describes the mechanism of neprilysin inhibitors in comparison to the specific features of ARNIs (671). ARNI is the combination of two drugs, i.e. an angiotensin receptor blocker and a neprilysin inhibitor. Consequently, the drug combines the mechanisms of two drugs in one tablet (Figure 5.1.7-1 and Figure 5.1.7-2).

![Figure 5.1.7-1 Formula of valsartan, an angiotensin II AT1 receptor blocker](image1)

![Figure 5.1.7-2 Formula of sacubitril, a prodrug of the neprilysin inhibitor LBQ657](image2)

Sacubitril is the novel medical agent included in ARNIs. Being a prodrug, sacubitril is activated after its resorption through the gut mucosa. The hepatic carboxyesterase-1 is the enzyme responsible for the drug’s metabolism into the active drug LBQ657, which inhibits the enzyme neprilysin (EC neutral endopeptidase). This enzyme is responsible for the breakdown of natriuretic peptides (NP), e.g. atrial natriuretic peptide (ANP) and b-type natriuretic peptide (BNP), which are synthesized and released by cardiomyocytes and have beneficial effects counteracting the development of heart failure. Neprilysin does also degrade angiotensin I and II. Thus, the neprilysin inhibition alone results in increased angiotensin II levels. To prevent the effects of accumulated angiotensin II, the inhibition of the angiotensin AT1 receptor with an angiotensin AT1 receptor blocker is a prerequisite for the use of neprilysin inhibitors.

The inhibition of neprilysin results in a reduced degradation of natriuretic peptides ANP, BNP and C-type natriuretic peptide (CNP). The activity of the natriuretic peptides is prolonged, resulting in several beneficial effects for patients with heart failure. Most effects of the natriuretic peptides counteract the effects of the renin angiotensin system, which are detrimental for the cardiovascular system. The natriuretic peptides bind to their NP receptors, namely NP receptor-A (NPR-A), NP receptor-B (NPR-B) and NP receptor-C (NPR-C). The binding of natriuretic peptide to NPR-A or NPR-B results in increased activity of the particulate guanylate cyclase, which produces cyclic guanosine monophosphate (cGMP). Thus, major
effects of the natriuretic peptides are caused by increasing intracellular cGMP levels. Natriuretic peptides can act on many cells of several organs.

The cGMP-dependent protein kinase in vascular smooth muscle cells is activated by increasing cGMP levels, finally resulting in relaxation of vascular smooth muscle cells. Consequently, systemic vascular resistance and blood pressure are lowered, leading to reduced afterload for the heart which is beneficial for the failing heart. By acting on renal epithelial tubule cells, the excretion of sodium and water through the kidneys is enhanced, cardiac preload and filling pressure are reduced. Natriuretic peptides also have antihypertrophic and antifibrotic effects on cardiomyocytes, partially by counteracting angiotensin II and endothelin-1. The effects of natriuretic peptides are further pronounced in patients with heart failure because they lack functional natriuretic peptides or develop resistance towards natriuretic peptides, e.g. by down-regulation of NP receptors.

Positive effects of physical activity on the cardiovascular system are partially linked to an increased release of atrial natriuretic peptide by the stretched cardiomyocytes. In patients with “elevated cardiac filling pressures”, cardiomyocytes are also mechanically stretched and release increased amounts of natriuretic peptides. Because cardiac filling pressures are elevated in heart failure, levels of natriuretic peptides are elevated in patients with heart failure and N-terminal pro-BNP (NT-proBNP) serves as a biomarker for heart failure. The generation or degradation of NT-proBNP is not influenced by ARNI, it can be used as a marker in patients taking ARNI.

The first compound on clinical trial with a neprilysin inhibitor, which resembled the pharmacological principle of ARNIs, was omapatrilat. In contrast to ARNIs, omapatrilat is only one pharmacologically active agent. Omapatrilat is multifunctional and inhibits the angiotensin converting enzyme (ACE), aminopeptidase P and neprilysine. This triple inhibition results in markedly decreased bradykinin degradation by these enzymes. As a consequence, clinical studies showed an increased frequency of angioedema induced by bradykinin in study patients. Omapatrilat was not approved by the FDA because the risk of angioedema was seriously elevated in patients taking it. In contrast, ARNIs do not inhibit the angiotensin converting enzyme and allow sufficient degradation of bradykinin, while the angiotensin II AT1 receptor is blocked by its second component, valsartan. Angioedema occurred much less frequent during treatment with ARNI than during treatment with omapatrilat (671).

**Treatment of patients with heart failure with preserved ejection fraction (PARAMOUNT trial)**

Heart failure with preserved ejection fraction is an increasing problem in the ageing population. Currently, about 50% of patients with heart failure have a preserved ejection fraction. Emerging risk factors for this type of heart failure are diabetes, obesity and ageing. So far no drugs have been officially approved for the treatment of HFpEF.

The effects of ARNI and valsartan on patients with symptomatic heart failure but preserved ejection fraction were compared in the PARAMOUNT trial, a phase II clinical trial (672). The patients suffered from symptoms of heart failure NYHA II or III, had an ejection fraction of at least 45% and showed elevated levels of NT-proBNP above 400 pg/ml. Before the trial started the study participants’ cardiac function and structure was assessed by echocardiography, the examination regularly showed an enlarged left ventricle. The primary point of the study was the reduction of NT-proBNP levels after 12 weeks of treatment. Considering the primary aim of reducing NT-proBNP values the ARNI compound LCZ696 (sacubitril/valsartan) achieved a significantly stronger reduction of NT-proBNP plasma levels than valsartan alone. This effect
shrunk after 36 months, the median NT-proBNP was still lower in the ARNI group but the difference was statistically not significant. Nevertheless, more patients in the ARNI treatment group showed substantial reduction of heart failure symptoms and switched to a lower NYHA class after 36 weeks. In accordance with the reduction of heart failure symptoms the echocardiographic assessment of the cardiac function after 36 weeks of therapy showed a reduction of atrial enlargement in the ARNI group, volume and dimension could be reduced to normal levels. No other echocardiographic measures, e.g. ejection fraction or left ventricular size changed. The improved echocardiographic outcome concerning the atria may reflect "reverse atrial remodeling". The ARNI was generally well-tolerated by the study population, the most frequent side effect of ARNI treatment was symptomatic hypotension, it occurred more frequently in the ARNI group than in the valsartan group. The risk for angioedema was not significantly elevated, only one patient suffered an angioedema of medium intensity in the ARNI group. The reduction of blood pressure was stronger in the ARNI group after 12 weeks (systolic reduction 9.3 mmHg, diastolic reduction 4.9 mmHg) than in the valsartan group (systolic reduction 2.9 mmHg, diastolic reduction 2.1 mmHg). After 36 weeks, the reduction of blood pressure became weaker for ARNI (systolic reduction 7.5 mmHg, diastolic reduction 5.1 mmHg) and valsartan (systolic reduction 1.5 mmHg, diastolic reduction 0.34 mmHg), possibly due to counter regulation by the renin angiotensin system or the sympathetic system. Statistical analysis showed that the stronger blood pressure lowering effect of ARNI was not responsible for the stronger reduction of NT-proBNP levels. Life quality, as measured by the the Kansas City Cardiomyopathy Questionnaire (KCCQ) score, showed no difference between the two groups. The study shows that the main risk is hypotension and that ARNI can reduce symptoms of heart failure more efficiently than valsartan in patients with heart failure with preserved ejection fraction (672).

Until now, according to the guidelines in place, only major risk factors for further development of heart failure and symptoms of heart failure are treated in heart failure with preserved ejection fraction. The dual inhibition of neprilysin and the angiotensin receptor blocker by ARNI showed beneficial effects on the development of heart failure itself in the PARAMOUNT study. ARNI may be the first drugs to be approved for the treatment of heart failure with preserved ejection fraction in the future. Patients for a larger phase III clinical trial are currently enrolled.

**ARNI reduces the mortality in patients with heart failure with reduced ejection fraction (PARADIGM-HF trial)**

The PARADIGM-HF trial compared the ACE inhibitor enalapril with the ARNI (sacubitril/valsartan) in the therapy of heart failure with reduced ejection fraction (673). Over 8000 patients were enrolled in the study. All of them had heart failure with an ejection fraction reduced below 40%. Only patients with symptomatic heart failure of NYHA class II, III or IV could participate. Another criterion for inclusion to the study were highly elevated plasma levels of BNP above 150 pg/ml or of NT-proBNP above 600 pg/ml. Enalapril was chosen as a reference substance because many studies had provided proof that enalapril reduced the mortality in patients with heart failure. Most participants were already taking standard medication according to the guidelines, including beta blockers, diuretics, angiotensin converting enzyme inhibitors or angiotensin receptor blockers. The composite primary endpoint consisted of cardiovascular mortality and hospitalization for heart failure symptoms, but study design allowed separate analysis of both primary endpoints.

ARNI showed to have such a positive effect on the primary outcome that the study had to be stopped prematurely for ethical reasons after the third interim analysis and before study completion (673). Study analysis showed that patients taking ARNI had a significantly
decreased cardiovascular mortality and a significantly lower rate for first time hospitalization due to heart failure symptoms in comparison to patients taking enalapril. The cardiovascular mortality could be reduced by over 3% in the ARNI group, while the risk for being admitted to hospital for symptoms of heart failure could even be reduced by 21%. All-cause mortality in patients taking enalapril was higher (19.8%) than in patients treated with ARNI (17%), which confirms the significance of reduced cardiovascular mortality in heart failure patients treated with ARNI. The follow up assessment after 27 months revealed that fewer patients taking ARNI (21.8%) reached the combined endpoint of cardiovascular death or first time hospitalization for heart failure than patients taking enalapril (26.5%).

Secondary endpoints of the study where time to death from any cause, life quality assessment through the reached score in the KCCQ, onset of atrial fibrillation and time to first symptoms of renal impairment. Treating patients with ARNI resulted in slower progression of heart failure and reduced symptoms of heart failure more effectively, when compared with enalapril treatment. The therapy with ARNI increased physical abilities of heart failure patients, in contrast to enalapril.

Some side effects occurred more frequently in patients under treatment with ARNI. The dual inhibition of the angiotensin receptor and neprilysin by ARNI resulted in significantly more cases of symptomatic hypotension and symptomatic severe hypotension with reduction of systolic blood pressure below 90mmHg. Angioedema also occurred more frequently in patients being under therapy with ARNI, but the difference between the groups was statistically insignificant.

The PARADIGM-HF trial showed that ARNI is more beneficial than the ACE inhibitor enalapril in the therapy of heart failure. Overall mortality, cardiovascular mortality and first time hospital admission rates were significantly reduced in heart failure patients taking ARNI, while enalapril showed weaker protective effects. At the same time, the study highlighted the most dangerous risk of therapy with ARNI, symptomatic hypotension. Care must be taken to administer ARNI in a dose not provoking hypotension, especially if combined with other antihypertensive acting agents like beta blockers or diuretics, which are regularly used for the treatment of heart failure. The safety of long term intake of ARNI still has to be evaluated. On the long run ARNI may replace ACE inhibitors and angiotensin receptor blockers for the therapy of heart failure in patients who tolerate it.

According to the new revised guidelines of the AHA and the ESC, ARNIs can replace the ACE inhibitor or the angiotensin receptor blocker in the therapy of heart failure with reduced ejection fraction, if symptoms are persistent under conventional heart failure therapy.
The major goal of this project was the investigation of pathomechanisms of heart failure to identify potential new targets for treatment. The project specifically focused on studying the impact of a moderately increased FASN (fatty acid synthase) level in the heart. This question relied on our previous data from a whole genome microarray gene expression study, which consistently found an elevated level of cardiac FASN in various models of experimental heart failure induced by major cardiovascular risk factors, i.e. chronic pressure overload and atherosclerosis (674). In addition, FASN was shown to be up-regulated in myocardial tissue samples from cardiac biopsies derived from patients with heart failure (674).

To study the role of FASN in the heart, I generated transgenic mice with myocardium-specific expression of the human FASN gene under control of the cardiomyocyte-specific promoter alpha-MHC. The FASN-transgenic mouse model should provide information about the role of an increased availability of lipids for heart function.

A second major goal consisted in the analysis of the functional network regulated by FASN. My whole genome microarray gene expression analysis identified major target genes of the transcription factor, Pparg, which were up-regulated by FASN. To elucidate underlying mechanisms, I generated and analyzed the phenotype of PPARG-transgenic mice.

Finally, because FASN-transgenic mice developed signs of heart failure, my goal was to find a treatment approach to counteract the effects of an increased FASN activity. We chose to analyze the effects of GRK2 inhibition, which is known to exert cardio-protection in several models of cardiac dysfunction and myocardial ischemia.
5.3 Publication "Inhibition of G-protein-coupled Receptor Kinase 2 Prevents the Dysfunctional Cardiac Substrate Metabolism in Fatty Acid Synthase Transgenic Mice"

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**Inhibition of G-Protein-Coupled Receptor Kinase 2 Prevents the Dysfunctional Cardiac Substrate Metabolism in Fatty Acid Synthase-Transgenic Mice**

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Running title: GRK2 inhibition retards cardiometabolic dysfunction

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**Keywords:** Cardiac metabolism, extracellular signal-regulated kinase (ERK), fatty acid synthase, G-protein-coupled receptor kinase 2 (GRK2; ADRBK1), heart failure, microarray, peroxisome proliferator-activated receptor (PPAR), transgenic mice

**5.3.1 Abstract**

Impairment of myocardial fatty acid substrate metabolism is characteristic of late-stage heart failure and has limited treatment options. Here we investigated whether inhibition of G-protein-coupled receptor kinase 2 (GRK2) could counteract the disturbed substrate metabolism of late-stage heart failure. The heart failure-like substrate metabolism was reproduced in a novel transgenic model of myocardium-specific expression of fatty acid synthase (FASN), the major palmitate-synthesizing enzyme. The increased fatty acid utilization of FASN-transgenic neonatal cardiomyocytes rapidly switched to a heart failure phenotype in an adult-like lipogenic milieu. Similarly, adult FASN-transgenic mice developed signs of heart failure. The development of disturbed substrate utilization of FASN-transgenic cardiomyocytes and signs of heart failure were retarded by the transgenic expression of GRKInh, a peptide inhibitor of GRK2. Cardioprotective GRK2 inhibition required an intact ERK (extracellular signal-regulated kinase) axis, which blunted the induction of cardiotoxic transcripts, in part by enhanced serine-273 phosphorylation of Pparg (peroxisome proliferator-activated receptor γ). Conversely, the dual-specific GRK2 and ERK cascade inhibitor, RKIP (raf kinase inhibitor protein), triggered dysfunctional cardiomyocyte energetics and the expression of heart failure-promoting Pparg-regulated genes. Thus, GRK2 inhibition is a novel approach that targets the dysfunctional substrate metabolism of the failing heart.
5.3.2. Introduction

Heart failure is a debilitating syndrome that involves insufficient cardiac performance. Multiple pathomechanisms have been elucidated but treatment options remain insufficient, and hence the mortality of heart failure is high (675). The causes of heart failure are complex with ischemic heart disease being the most frequently associated condition (538). Co-existing disorders such as diabetes, hypertension and obesity, further deteriorate symptoms (676). Despite having a different aetiology, late-stage heart failure is commonly characterised by severe changes in myocardial substrate metabolism, with a switch from fatty acid oxidation towards predominant glycolysis (677) (678) (679). Conflicting evidence exists as to whether this substrate switch is beneficial or detrimental (680), but several previous studies have indicated that an increased availability of lipid substrates that counteract the substrate switch could improve cardiac function (680) (681). Moreover, treatment options, which improve substrate availability, are attractive because the failing heart is often considered to be “an engine, running out of fuel” (619).

Following this concept, we aimed to investigate the impact of improved cardiac substrate availability by generating transgenic mice with myocardium-specific expression of fatty acid synthase (FASN), the major palmitate-synthesizing enzyme. Such an approach is also supported by data obtained for myocardium-specific Fasn deficiency, which have revealed the cardioprotective potential of Fasn (682). On the other hand, hearts from patients with heart failure showed an increased expression and protein level of FASN (674) (682). By generation of transgenic mice, we found that FASN-transgenic mice developed a heart failure-like phenotype with impaired cardiomyocyte substrate use. In search for a treatment approach for the disturbed cardiac substrate metabolism, we focused on the role of GRK2 inhibition because GRK2 inhibition could counteract cardioprotection by promotion of a cardiomyocyte survival program (512) (656), and resensitization of the cardioprotective adiponectin receptor 1 (683) (684) (685). For GRK2 inhibition in vivo, we used GRKInh, a small peptide inhibitor derived from the first intracellular loop of the β2-adrenergic receptor (512) (686). Our data with GRKInh show that GRK2 inhibition counteracts the heart failure-related cardiac metabolic dysfunction and signs of heart failure of FASN-transgenic mice.

5.3.3 Experimental Procedures

5.3.3.1. Generation of transgenic mice and animal experiments

Transgenic mice were generated as described (512) with minor modifications. Briefly, for the generation of transgenic mice with myocardium-specific expression of FASN, we constructed a transgene that placed the FASN cDNA under the control of the α-MHC (α-myosin heavy chain) promoter (512). For the generation of transgenic mice with myocardium-specific expression of UCP1 (uncoupling protein-1), PPARG and PPARG-S273A (isoform-1, which lacks amino acids 1-28 of isoform-2; serine-273 refers to the numbering of isoform-2) a similar approach was used. The plasmid sequence was removed by NotI digestion, and the purified linear DNA (2 ng/μl) was injected into fertilised oocytes of superovulated B6 (C57BL/6J) mice. For generation of transgenic Tg-PPARG and Tg-PPARG-S273A mice, fertilized oocytes from non-transgenic and Tg-GRKInh mice (transgenic mice with myocardium-specific expression of GRKInh, a GRK2-specific peptide inhibitor with the peptide sequence MAKFERLQTVTNYFITSE) were used. Transgenic mice with myocardium-specific expression of GRKInh or human RKIP (PEBP1) were generated and characterized previously (12). Mouse lines in the study were deposited into the JAX repository (The Jackson Laboratory, USA) and have the following strain ID numbers: 911818 [C57BL/6Tg(MHCPEBP1)1 Sjaa];
The effect of rosiglitazone-induced Pparg activation was analysed with 8-month-old male ApoE/- mice, which had received 30 mg/kg/d rosiglitazone for two months. Untreated, age-matched ApoE-/-, and non-transgenic B6 mice served as control groups. Abdominal aortic constriction (AAC) was performed in 4-month-old male B6 mice to trigger pressure overload-induced cardiac hypertrophy and signs of heart failure (674). Age-matched control mice underwent the identical surgical procedure except for ligation of the aorta (sham-operated mice). All of the mice were kept on a 12 h light/12 h dark regime, and had free access to food and water. The ApoE-/- mice were fed a rodent chow that contained 7 % fat and 0.15 % cholesterol (AIN-93-based diet) whereas B6 mice were fed a standard rodent chow containing 4.5 % fat.

Transthoracic echocardiography was performed with a Vivid 7 echocardiograph equipment (GE Healthcare) with a 12 MHz linear array transducer similarly as previously described (674). The left ventricular ejection fraction was calculated in the M-mode of the parasternal long-axis view using the formula of Teichholz. Recordings were interpreted offline using EchoPac Pc 3.0 software (GE).

Animal experiments were performed in accordance with the NIH guidelines, and reviewed and approved by the local committee on animal care and use (University of Zurich).

5.3.3.2 Whole genome microarray gene expression analysis

Whole genome microarray gene expression analysis of cardiac tissue was performed using Affymetrix GeneChip Mouse genome MG430 2.0 Arrays essentially as described previously (687). GO (gene ontology) analyses of microarray data were performed with GCOS and/or RMA-processed data using GeneSpring GX software (Agilent). Probe sets, which were significantly up-regulated in failing hearts (fold change ≥ 2 relative to the respective control group and \( P \leq 0.01 \)) were used for GO classification. Microarray gene expression data are available at the NCBI GEO database accession numbers GSE25765-8 (GSE25765, GSE25766, GSE25767, GSE25768), GSE28031 and GSE49351.

Gene expression of selected genes was also analysed by real-time quantitative (q) RT-PCR with a LightCycler 480 (Roche Diagnostics).

Sequences of the forward and reverse primers were as follows:

\begin{align*}
\text{Acaca forward} & \ 5'\text{G CCTCCGTACGCTACGATAC}3' \\
\text{Acaca reverse} & \ 5'\text{G ACCACCCGACGATCG}3' \\
\text{Adipoq forward} & \ 5'\text{ACTGCAACATTCCGGGACTC}3' \\
\text{Adipoq reverse} & \ 5'\text{GAGGCCTGGTCCACATTCTT}3' \\
\text{Fasn forward} & \ 5'\text{GGCCCCTCTGTTAATTGGCT}3' \\
\text{Fasn reverse} & \ 5'\text{CGCTTGTTGGTGGACACTTG}3' \\
\text{FASN forward} & \ 5'\text{TGGGCTCTGTTAATTGGCT}3' \\
\text{FASN reverse} & \ 5'\text{AGCCAGTGTGCTGTCGAT}3' \\
\text{PPARG forward} & \ 5'\text{GCTCCGTGGATCTCTCCGTA}3'
\end{align*}
PPARG reverse 5’-AGCTTTATCTCCACAGACACGA-3’;

Retn forward 5’-GTCCTGCTAATCTCTGACACGA-3’;
Retn reverse 5’-GGCTGCTGTCCAGTCTATCCTT-3’;

Ucp1 forward 5’-CACTGCAAAGTCCGCTTCAG-3’;
Ucp1 reverse 5’-GCAGGCGAGCCGCTCAGTT-3’.

**Lentiviral-mediated down regulation of Fasn and Ucp1 by RNAi in vivo**

For the down regulation of Fasn expression in vivo, ApoE−/− mice were transduced by i.p. administration of a replication-incompetent lentivirus (1x10⁸ copies/mouse in PBS), which down-regulates Fasn by polymerase II (Pol II)-dependent expression of a pre-miRNA targeting the Fasn RNA by RNAi. Endogenously expressed Ucp1 was down-regulated by the transduction of B6 mice with a lentivirus that expressed a pre-miRNA targeting Ucp1 by RNAi. The lentiviral expression plasmids were generated by inserting the indicated double-stranded oligonucleotides that encoded an engineered pre-miRNA sequence into the pLenti6/V5-Dest Gateway® Vector (Invitrogen):

miFasn top strand
5’-TGCTGATAACTGAGTGTTTGTGGTGGCCACTGACTGACAAGAC CCGTCCAAGTTAT-3’;

miFasn bottom strand
5’-CCTGATAACTGGAGCGGGTGCTGGTCAGTCAGTGCCAAAACAAGACCCGA ACTCCAAAGTTATC-3’;

miUcp1 top strand
5’-TGCTGTGTATCCCATGCAGATGGCTGTGCTTGGCCACTGACAGCCATCT ATGGGATCAA-3’;

and

miUcp1 bottom strand
5’- CCTGTTTGATCCCATAGATGGCTGTGCTGTCAGTCAGTGCCAAAACAGCCATCTGCAT GGGATCAAAC-3’.

A pseudotyped lentivirus was produced by cotransfection of 293FT cells with the lentiviral plasmid and a mixture of packaging plasmids pLP1, pLP2 and pLP/VSVG (Invitrogen). To quantify the lentivirus integration in vivo, we used primers that comprised sequences that were derived from the cytomegalovirus immediate early promoter and the pre-miRNA-sequence. Down regulation of Fasn (or Ucp1) expression was confirmed by real-time qRT-PCR after the transduction of mice or isolated neonatal mouse cardiomyocytes with miFasn-lentivirus (or miUcp1-lentivirus).

**5.3.3.3 Antibodies**

The following antibodies were used for immunohistochemistry, immunofluorescence and immunoblotting: anti-ARRB1 (beta-arrestin-1) antibodies, raised in mouse against recombinant ARRB1 (512); anti-Agrt1 (AT1R) antibodies, which were raised in rat against the carboxyl-terminal region of Agrt1 (688); anti-FASN antibodies, which were raised in rabbit.
against an antigen encompassing amino acids 2205-2504 of FASN (674); anti-GRK2 (ADRBK1) antibodies, raised in rabbit against recombinant GRK2 protein (512); anti-GRK5 antibodies, raised in rabbit against recombinant GRK5 protein (512); anti-GRKinhib antibodies, raised in rabbit against GRKinhib (512); anti-Pparg antibodies, raised in rabbit against an antigen encompassing amino acids 8-106 of Pparg (Santa Cruz Biotechnology Inc., USA) or synthetic phosphopeptides derived from PPARG around the phosphorylation site of S273 or S112 (BIOSS antibodies; Abcam); and Ucp1/Ucp1 antibodies were raised in rabbit against an antigen encompassing amino acids 288-302 of mouse/human Ucp1/UCP1 (674). For the immunohistological and immunoblot detection of activated phospho-ERK1/2, phospho-ERK1/2-specific antibodies were used detecting activated ERK1/2 phosphorylated at T202+Y204 of ERK1, and T185+Y187 of ERK2 (E10 mouse mAb, Cell Signaling). For immunoblot detection of ERK1/2 ERK1/2-specific antibodies raised in rabbit (Cell Signaling) were used, and immunofluorescence detection of p38 MAPK (mitogen-activated protein kinase) on cardiac sections was performed with anti-p38 antibodies (Cell Signaling). The immunoblot detection of activated AMPKα (Prkaa1/2; protein kinase, AMP-activated alpha 1/2 catalytic subunit) phosphorylated on T183/172 was detected with antibodies raised in rabbit against a synthetic peptide corresponding to residues that surrounded T172 (40H9, Cell Signaling). Immunoblotting and immunohistochemistry were routinely used to determine and confirm the cross-reactivity of the antibodies with the respective mouse and human proteins.

5.3.3.4 Immunohistology analyses and immunofluorescence

For immunohistology, we used paraffin sections or cryosections of mouse heart specimens. Immunohistological detection of Fasn (FASN) was performed with affinity-purified, polyclonal antibodies as described (674). Methods describing oil red O staining, immunofluorescence detection of proteins, and immunohistology for activated phospho-ERK1/2 in paraffin sections or cryosections have been described previously (512) (674) Immunohistology sections were imaged with a Leica DMI6000 microscope equipped with a DFC420 camera, and immunofluorescence imaging was performed with a Leica (TCS) confocal laser microscope.

5.3.3.5 Immunoblot detection of proteins

For immunoblot detection of proteins, cardiac tissue was pulverized in liquid nitrogen and extracted with RIPA buffer supplemented with protease/phosphatase inhibitor cocktail, as previously described (470). Detection of proteins was performed with affinity-purified antibodies or F(ab)2 fragments of the respective antibodies (512) (674) after separation of proteins by SDS-PAGE and subsequent electrophoretic protein transfer to PVDF membranes. Bound antibody was visualised with F(ab)2 fragments of enzyme-coupled secondary antibodies (Dianova), or by enzyme-coupled protein A (Merck Millipore) as applicable, and was followed by enhanced chemiluminescent detection (ECL Prime, Amersham).

5.3.3.6 Functional assays

Mouse or rat neonatal cardiomyocytes were isolated and transfected as described (512) (689). Fibroblasts were removed by preplating for 1 h at 37°C. Cardiomyocytes were collected and cultivated in MEM [minimum essential medium supplemented with 5 % FCS and 25 mg/l BrdU (5-bromo-2’-deoxyuridine)]. For knockdown of Fasn and Ucp1, neonatal cardiomyocytes were transfected with stealth RNAi targeting the coding sequence of rat or mouse Fasn (nucleotides 428-452 and 1990-2014; Invitrogen) and Ucp1 (nucleotides 289-313 and 401-425; Invitrogen). For cardiomyocyte expression of PPARG and PPARG-S273A, the human cDNAs encoding PPARG and PPARG-S273A, were inserted into the KpnI/XbaI sites
of pcDNA3 (Invitrogen). All of the mutants and constructs that were generated by PCR were sequenced entirely. DNA strand breaks were determined in situ by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique (Roche Applied Science) (512). Measurement of the [ATP] of cardiac tissue extracts was performed as described (674), and Pparg transcription factor DNA binding activity was determined with a Pparg transcription factor assay kit (Abcam). Cellular cAMP, total cardiac free fatty acids (FFA) and triacylglycerol (TAG) contents were analysed as detailed previously (674) (689). Cardiac contents of diacylglycerol (DAG) and ceramides were determined with the DAG kinase assay method as described (690).

5.3.3.7 Measurement of cardiomyocyte substrate metabolism

We used a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) to determine the cardiomyocyte substrate metabolism. The oxygen consumption rates, OCR (pmol/min), and extracellular acidification rates, ECAR (the H+ production rate, mPH/min), of neonatal cardiomyocytes (10 000 cells/well) plated on Cell-Tak-coated plates (Discovery Labware Inc., Bedford, MA, USA) were measured in assay medium (i.e., unbuffered DMEM supplemented with 5.5 mM glucose and 0.5 mM carnitine) according to the Installation and Operation Manual from Seahorse Bioscience. The oxidation of endogenous fatty acids (without exogenously added palmitate, to detect the function of transgenic FASN expression) was determined by measurement of the absolute and relative OCR that was inhibited by the CPT-1 (carnitine palmitoyl transferase 1) inhibitor, Etomoxir (50 µM). The extent of glycolysis was determined by measurement of the absolute and relative ECAR, which was inhibited by 50 mM 2-deoxyglucose (2-DG). As indicated, we also determined the effect of an adult-like lipogenic milieu by the cultivation of cardiomyocytes for 10 days with a 3F protocol that consisted of insulin (5 µg/ml), 3-isobutylmethylxanthine (IBMX; 0.25 mM) and dexamethasone (0.5 µM) (691), which were added as supplements to the standard medium. As a control for the 3F protocol, we used cardiomyocytes that were cultivated for 10 days under standard conditions. Long-term cultivation of neonatal cardiomyocytes is a model of in vitro senescence characterized by metabolic deficiencies (692), which could account for the overall low β-oxidation rate of 5-25 % in our experiments. Metabolic flux experiments were performed on 6 wells of a 24-well plate (technical replicates), and were reproduced at least three times (biological replicates). The oligomycin-insensitive OCR (a measure of mitochondrial uncoupling) was determined after the addition of 2.5 µM oligomycin. The non-mitochondrial OCR that remained after the addition of rotenone/antimycin A (2 µM) was subtracted.

5.3.3.8 Statistical analyses

The results are presented as the means ± SD unless otherwise specified. The P values were calculated with Student’s t-test. Analysis of variance was performed for comparisons between more than two groups followed by a post-test (Tukey’s multiple comparison test unless otherwise specified), and statistical significance was set at a P value of <0.05 unless otherwise stated.
5.3.4 Results

5.3.4.1 FASN-transgenic cardiomyocytes developed a dysfunctional cardiac substrate metabolism

A dysfunctional cardiac substrate metabolism is a common feature of late-stage heart failure with limited treatment options. To reproduce the energy substrate use of heart failure patients and experimental models, which commonly show up-regulation of the major palmitate-synthesizing enzyme, FASN (674) (682), we generated a transgenic model with myocardium-specific FASN expression under the control of the α-MHC-promoter (Fig. 1A, B). Immunoblot detection of the FASN protein confirmed transgenic protein expression in hearts from mice with stable genomic integration of the FASN transgene whereas the Fasn protein was barely detectable in non-transgenic B6 hearts (Fig. 1C). Two different transgenic lines (derived from founders no. 3 and 9) were established, which showed comparable FASN protein levels (Fig. 1C). All of the experiments were independently performed with these two transgenic lines.

Figure 1 - FASN-transgenic cardiomyocytes developed a dysfunctional cardiac substrate metabolism. (A) Scheme of the α-MHC-FASN vector used for the generation of FASN-transgenic mice. (B) Identification of the α-MHC-FASN transgene in the genomic DNA of different founder mice by PCR. The asterisks denote the amount of the FASN transgene-specific PCR product [***, ***, *, >, =, < amount of PCR product obtained with 2 ng of plasmid template (P)]. (C) Immunoblot detection of the FASN protein in hearts from different founder mice with FASN-specific antibodies (IB: FASN). The asterisks indicate the amount of transgenic FASN protein relative to the non-transgenic control after normalization to Gnb [***, 2.5-fold; **, 2.3-fold; *, 1.8-fold (no. 5) and 1.4-fold (no. 10) increase over control]. The lower panel shows a control immunoblot detecting Gnb.

After the generation of the transgenic mouse lines, the metabolic energetics of isolated neonatal FASN-transgenic (Tg-FASN) cardiomyocytes was determined with a Seahorse Bioscience XF24 Extracellular Flux Analyzer. We measured the β-oxidation of endogenous fatty acids by the Etomoxir-sensitive fraction of the oxygen consumption rate (OCR), and glycolysis by the 2-deoxyglucose-sensitive extracellular acidification rate (ECAR) (Fig. 2A, B). Compared to non-transgenic controls, FASN-transgenic cardiomyocytes showed significantly more β-oxidation under basal conditions (22.7±1.5% vs. 5.3±0.9%; Fig. 2C, D). This finding indicated that FASN increases the substrate availability of cardiomyocytes for β-oxidation.
We next determined the effect of an adult-like lipogenic milieu on neonatal cardiomyocytes by treatment with a 3F protocol consisting of insulin, IBMX, and dexamethasone for 10 days (691). In control cardiomyocytes, the 3F protocol switched the embryonic-like metabolism dominated by glycolysis (693) to an adult-like metabolism, which was characterized by an increased baseline OCR (Fig. 2B), and more fatty acid β-oxidation (Fig. 2D). In contrast, FASN-transgenic cardiomyocytes developed a heart failure-like phenotype with an overall depressed substrate metabolism (Fig. 2B), and predominant glycolysis (Fig. 2C). Thus, the increased availability of the lipid substrate, palmitate, did not protect neonatal cardiomyocytes from a substrate switch to a heart failure-like phenotype that had depressed bioenergetics dominated by glycolysis.
5.3.4.2 FASN-transgenic mice developed signs of heart failure and cardiac lipid load

The extracellular flux analysis detected a heart failure-like metabolic substrate use in isolated cardiomyocytes from Tg-FASN mice. But the method employs unloaded cardiomyocytes and does not represent the condition in the loaded myocardium, which functions with real heart rates. Therefore, we analysed the cardiac phenotype in vivo. Our data show that adult FASN-transgenic mice developed signs of heart failure as early as 6 months of age, as evidenced by a significantly decreased left ventricular ejection fraction, cardiac hypertrophy with dilatation, and increased cardiomyocyte apoptosis (Fig. 3A-E). As a control, the body weight of 6-month-old Tg-FASN mice was not different from B6 control mice (i.e., 34.28±1.02 g and 33.45±0.98 g, respectively; ±s.d.; n=4; P=0.5909).

![Figure 3 - FASN-transgenic mice developed signs of heart failure and cardiac lipid load.](image)

(A) Decreased left ventricular ejection fraction of 6 month-old Tg-FASN mice. (B) Histological analysis of a Tg-FASN heart compared to a B6 heart (bar: 2 mm). (C) Increased heart-weight-to-body-weight ratio of Tg-FASN mice. (D, E) Increased number of TUNEL-positive cardiomyocytes of Tg-FASN hearts. Panel (E) shows representative immunohistological sections (bar: 20 µm). The data shown are the mean ±SD, n=5; ***,P<0.0001 (A, C, D). Histology experiments are representative of four different mice/group (B).
The cardiac FASN protein in FASN-expressing mice was detected by immunoblotting and immunohistology (Fig. 4A-C). The FASN protein level in Tg-FASN hearts was increased ~2.4-fold (Fig. 4A, B), which is comparable to the up-regulated FASN level of failing human hearts (674) (682).

Concomitantly to the increased FASN protein, cardiac free fatty acid (FFA) and triacylglycerol contents of Tg-FASN mice with signs of heart failure were elevated 2.2-fold and 2.1-fold, respectively (Fig. 5A, B). The cardiac contents of diacylglycerol (DAG) and ceramides, which can be induced by palmitate, i.e., the major lipid synthesized by FASN, were also significantly increased (Fig. 5C, D). These lipids could be involved in the heart failure phenotype of Tg-FASN mice because they trigger a wide spectrum of cardiotoxic mechanisms, which involves, e.g., the excessive formation of reactive oxygen species, an increased endoplasmic reticulum stress, enhanced apoptosis, and mitochondrial dysfunction (690) (694). Additionally, increased cardiac contents of DAG and ceramide could mediate the activation of protein kinase C (PKC), which further decreases heart function (690) (695) (696). Thus, FASN-transgenic hearts developed signs of heart failure with cardiotoxic lipid load in addition to the dysfunctional energy substrate metabolism, which was detected in isolated cardiomyocytes.

**Figure 4** - Detection of the cardiac FASN protein levels by immunoblotting (A,B) and immunohistology (C) relative to the B6 control (bar: 40 μm). Panel (A) shows quantitative data that were obtained by densitometric scanning of immunoblots (±SD, n=4; ***P=0.0005) Histology experiments are representative of four different mice/group (C).

**Figure 5** - The cardiac free fatty acid, FFA (A), triacylglycerol, TAG (B), diacylglycerol, DAG (C), and total ceramide (D) contents of Tg-FASN mice relative to non-transgenic B6 controls [± SD, n=5; ***P=0.0006 (A), 0.0004 (B) and 0.0001 (C); **P= 0.0028 (D)].
5.3.4.3 Up-regulation of the heart failure-related cardiac lipid metabolic process in FASN-transgenic mice

Whole genome microarray gene expression profiling further confirmed the heart failure phenotype of Tg-FASN hearts by demonstrating the significant up-regulation of the heart failure-related cardiac lipid metabolic process (Fig. 6, Upper Panel).

A similar induction of those adipogenic genes was also observed when signs of heart failure were triggered by 6 months of pressure overload (674) (Fig. 6, Lower Panel). In contrast, cardiac hypertrophy, without signs of heart failure (674) and induced by 4 weeks of pressure overload, did not up-regulate the heart failure-related adipogenic gene expression signature (Fig. 7). Moreover, cardiac hypertrophy in the absence of heart failure signs promoted a significantly decreased expression of two probe sets that detect two enzymes of fatty acid biosynthesis, i.e., *Scd1* (Stearoyl-CoA desaturase-1) and *Acly* (ATP citrate lyase) (Fig. 7). Thus, a heart failure-related adipogenic gene expression signature accompanied the onset of heart failure signs in FASN-transgenic mice.
Heart failure-related adipogenic genes triggered by FASN are Pparg targets

In search of FASN-induced pathomechanisms, we focused on the adipogenic and heart failure-promoting transcription factor, Pparg (697) (698), because (i) palmitate, the major lipid synthesised by FASN, enhances the activity of Pparg (699), and (ii) adipogenic genes induced by FASN are Pparg targets (700), which are similarly up-regulated by Pparg over-expression in the mouse heart (697). Similarly, the treatment of ApoE−/− mice for 2 months with the Pparg agonist, rosiglitazone, also significantly up-regulated those heart failure-related adipogenic Pparg target genes, which were induced by FASN (Fig. 8). As a control, rosiglitazone promoted signs of heart failure in ApoE−/− mice (cf. Fig. 10A-D). These findings demonstrate that the heart failure-related adipogenic gene signature induced by Tg-FASN and chronic pressure overload is also triggered by direct Pparg activation with rosiglitazone.

Figure 7 - Adipogenic genes were not significantly up-regulated in 5 month-old B6 mice with cardiac hypertrophy induced by 1 month of ACC (Cardiac hypertrophy) in the absence of heart failure signs compared to the sham control (Sham-1mo). The statistical significance of the microarray data was evaluated using signal intensity values of probe sets (*,P<0.05; **,P≤0.01; ***,P<0.001 vs. the respective control; ±SD, n=2 gene chips per group, cRNA pooled from 4 mice/gene chip).

Figure 8 - Significantly up-regulated genes of the lipid metabolic process in hearts of 8-month-old ApoE−/− mice with 2 months of Pparg activation with rosiglitazone compared to untreated ApoE−/− mice. The probe sets were categorized into genes involved in lipid synthesis, storage, oxidation (Oxid.), and adipocyte differentiation (Adipoc.). The statistical significance of the microarray data was evaluated using signal intensity values of probe sets (*,P<0.05; **,P≤0.01; ***,P<0.001 vs. the respective control; ±SD, n=2 gene chips per group, cRNA pooled from 4 mice/gene chip).
5.3.4.5 Down regulation of endogenous Fasn reveals a causal relationship between Fasn and Pparg activation in promoting cardiac dysfunction

Next we investigated the impact of Fasn on Pparg-induced cardiac dysfunction, and we knocked down the endogenously expressed Fasn by RNAi in rosiglitazone-treated ApoE-/- mice.

**Figure 9** - Down regulation of endogenous Fasn reveals a causal relationship between Fasn and Pparg activation in promoting cardiac dysfunction. **(A, B)** Stable integration of lentiviral miFasn-DNA into the genomic DNA of ApoE-/- mouse hearts (H1, H2), kidneys (K1, K2) and livers (L1, L2) isolated two months after lentiviral transduction. Control DNA (cont. DNA) was isolated from an ApoE-/- mouse. In (B), the genomic integration of lentiviral DNA was quantified by qPCR (±SD; n=6; ***,P<0.001; *,P<0.05 vs. Heart). **(C)** Lentivirus-mediated delivery of miFasn decreased the expression of Fasn in hearts of ApoE-/- mice with 2 months of Pparg activation by rosiglitazone (±SD; n=3; ***,P=0.0029). **(D,E)** Immunohistological detection of Fasn (D) with anti-Fasn antibodies (anti-Fasn), and total lipids by oil red O staining (E) in cardiac sections of ApoE-/- mice after 2 months of Pparg activation and transduction of a control lentivirus (miCon) or lentiviral delivery of miFasn (bar: 40 μm). Histology experiments are representative of four different mice/group (D, E).

Inhibition of Fasn by lentiviral transduction of a Fasn-specific miRNA (Fig. 9A-D) retarded the development of rosiglitazone-induced cardiac lipid load (Fig. 9E) and signs of rosiglitazone-induced heart failure (Fig. 10A-D). Together these data confirmed the causal relationship between Fasn and enhanced Pparg activation in promoting cardiotoxicity and cardiac dysfunction.
5.3.4.6 GRK2 inhibition by GRKInh in FASN-transgenic mice

In view of the central role of FASN, the inhibition of FASN would be a straightforward treatment approach. However, FASN is an essential enzyme that has indispensable functions in energy homeostasis, membrane biology and neurogenesis, which preclude long-term FASN inhibition in vivo (682) (701) (702). We therefore searched for an alternative strategy to target the dysfunctional cardiac substrate metabolism. We focused on the inhibition of GRK2, which is a well-established means of cardioprotection (512) (703). Furthermore, GRK2 inhibition enhances the ERK cascade (512), which promotes (partial) inactivation of Pparγ (704). In agreement with heart failure patients (705), the GRK2 protein levels were significantly upregulated (i.e., 1.81±0.12-fold) in Tg-FASN hearts with signs of heart failure relative to the B6 controls (Fig. 11).
To inhibit GRK2 in vivo, we used a GRK2-specific inhibitor peptide (GRKInh) derived from the first intracellular loop of the β2 adrenergic receptor (512) (686). We used transgenic mice with myocardium-specific expression of GRKInh, which were established previously (512). The GRKInh peptide interacted specifically with GRK2 in heart tissue extracts from Tg-GRKInh/FASN mice as demonstrated by co-enrichment whereas the amount of GRK5 co-enriched with GRKInh-specific antibodies was below the limit of detection (Fig. 12).

Figure 11 - GRK2 inhibition by GRKInh in FASN-transgenic mice. Cardiac up-regulation of the GRK2 protein level in Tg-FASN relative to B6 hearts was detected by immunoblotting with GRK2-specific antibodies (n=4 mice/group). The lower panel is a control immunoblot detecting Gnb. The right panel shows the quantitative immunoblot evaluation (±SD, n=4).

Figure 12 - Immunoaffinity enrichment of GRKInh (AP, +) with GRKInh-specific antibodies from Tg-GRKInh/FASN hearts, and immunoblot detection (IB) of co-enriched GRK2 protein (left panel) and GRK5 protein (right panel). The control experiment (AP, -) applied an affinity matrix with immobilised control IgG. The lower panels show immunoblot detection of enriched GRKInh.
Quantitative assessment of the GRK2-GRKInh interaction indicated that 83.6±4.2 % of the total cardiac GRK2 protein was captured by GRKInh-affinity enrichment whereas the amount of GRK5 protein bound to GRKInh was less than 20% (i.e., 18.9±2.2%) of the total cardiac GRK5 content (Fig. 13).

The functional effects of GRK2 inhibition in transgenic Tg-GRKInh/FASN hearts were analyzed by the immunofluorescence detection of Arrb1, which translocates to phosphorylated membrane-spanning receptors as a direct consequence of GRK2-mediated phosphorylation (Fig. 14). In agreement with an increased GRK2 activity, immunofluorescence analysis detected the substantial membrane localization of Arrb1 in a cardiac section from a Tg-FASN mouse with signs of heart failure (Fig. 14, left panel). In contrast, the double-transgenic Tg-GRKInh/FASN heart section showed a largely cytoplasmic localization of Arrb1 as evidenced by co-localization with the cytosolic p38 MAPK (Fig. 14, right panel). These findings indicate that GRKInh interacts with GRK2 in hearts from double-transgenic Tg-GRKInh/FASN mice. As a consequence of the GRKInh-GRK2 interaction, the enhanced GRK2-mediated Arrb1 membrane translocation could be blunted.
5.3.4.7 GRK2 inhibition by GRKInh prevents the dysfunctional cardiac energetics of FASN-transgenic cardiomyocytes

We characterised the substrate metabolism of isolated neonatal cardiomyocytes from double-transgenic Tg-GRKInh/FASN mice compared to single-transgenic Tg-FASN mice. Under basal conditions, the presence of GRKInh retarded the premature appearance of an adult-like metabolic phenotype with an increased baseline OCR of Tg-FASN cardiomyocytes (Fig. 15).

**Figure 14** - Immunofluorescence co-localization of Arrb1 with p38 MAPK in cardiac sections from Tg-FASN and Tg-GRKInh/FASN mice (bar: 20 μm). Arrb1 was detected with affinity-purified mouse anti-Arrb1 antibodies followed by F(ab)2 fragments of Alexa Fluor 546-labelled (red) secondary antibodies, and p38 was detected with affinity-purified rabbit anti-p38 MAPK antibodies followed by F(ab)2 fragments of Alexa Fluor 488-labelled (green) secondary antibodies. Cell nuclei were stained with DAPI (blue). Immunofluorescence experiments are representative of four different mice/group.
Concomitantly, the increased β-oxidation of Tg-FASN cardiomyocytes under basal conditions was also retarded in double-transgenic mice that co-expressed the GRK2-inhibitor, GRKInh. Real-time measurements of OCR and ECAR of neonatal cardiomyocytes isolated from FASN-transgenic (Tg-FASN), double-transgenic Tg-GRKInh/FASN and non-transgenic B6 mice were performed under basal conditions (left panels) and after treatment with the 3F protocol (right panels). The OCR and ECAR values normalised to the baseline (A), and the absolute values of OCR and ECAR (B) are presented. The data are shown as the means ± SD; n=6 technical replicates (A, B).

Figure 15 - GRK2 inhibition by GRKInh prevents the dysfunctional cardiac energetics of FASN-transgenic cardiomyocytes. (A, B) The development of a dysfunctional cardiomyocyte energetics of FASN-transgenic cardiomyocytes was retarded in double-transgenic mice that co-expressed the GRK2-inhibitor, GRKInh. Real-time measurements of OCR and ECAR of neonatal cardiomyocytes isolated from FASN-transgenic (Tg-FASN), double-transgenic Tg-GRKInh/FASN and non-transgenic B6 mice were performed under basal conditions (left panels) and after treatment with the 3F protocol (right panels). The OCR and ECAR values normalised to the baseline (A), and the absolute values of OCR and ECAR (B) are presented. The data are shown as the means ± SD; n=6 technical replicates (A, B).

The improved metabolic profile of Tg-GRKInh/FASN cardiomyocytes was accompanied by a resensitization of adiponectin receptor protein 1 (Adipor1)-mediated signaling (Fig. 17), which is desensitized by GRK2 in the ischemic heart (683). Notably, the inhibition of GRK2 in Tg GRKInh/FASN cardiomyocytes led to a significantly increased protein level of activated phospho-Prkaa upon adiponectin stimulation whereas the adiponectin-stimulated signal was largely blunted in Tg-FASN cardiomyocytes (Fig. 17). This finding could be relevant because Adipor1 and its target AMP-activated protein kinase (Prkaa) could protect against palmitate-induced toxicity (684) (685).
Figure 16 - (A-C), The Etomoxir-blocked fraction of OCR, which represents fatty acid β-oxidation (A), the 2-deoxyglucose-blocked fraction of ECAR, which represents glycolysis (B), and the ratio of glycolysis/β-oxidation (C) were determined with cardiomyocytes isolated from Tg-FASN, Tg-GRKInh/FASN, B6 and Tg-GRKInh mice. The data are shown as the means ±SD; n=3 biological replicates (16 A-C); *, P<0.05, **, P<0.01 and ***, P<0.001 vs. Tg-FASN.

Figure 17 - Cardiomyocytes from Tg-GRKInh/FASN (lanes 3,4) mice showed significant adiponectin (Adipoq)-stimulated activation of Prkaa relative to Tg-FASN cardiomyocytes (lanes 1,2). Cardiomyocytes (3F protocol) were stimulated without (-) and with (+) globular domain adiponectin (2 μg/ml) for 60 min, and the activation of Prkaa was determined by immunoblotting with phospho-Prkaa- (p-Prkaa)-specific antibodies. The left panel presents quantitative data from 4 independent experiments (±SD; n=4; ***, P<0.0001), and the middle and right panels show representative immunoblots.
5.3.4.8 GRK2 inhibition retards the development of heart failure signs in Tg-FASN mice

The improved substrate metabolism upon GRK2 inhibition was also reflected in vivo, in adult murine hearts. The presence of GRKInh in double-transgenic Tg-GRKInh/FASN hearts compared to single-transgenic Tg-FASN hearts led to a significantly decreased total FFA and triacylglycerol load compared to single-transgenic Tg-FASN hearts (Fig. 18).

![Figure 18](image)

**Figure 18** - GRKInh retards the development of heart failure signs in Tg-FASN mice and promotes ERK axis-dependent inhibition of Pparg transcriptional activity. Cardiac free fatty acid, FFA (left panel), and triacylglycerol, TAG (right panel), contents of Tg-FASN mice relative to double-transgenic Tg-GRKInh/FASN, non-transgenic B6, and single-transgenic Tg-GRKInh mice (±SD, n=5; *, P<0.05 vs. Tg-FASN and Tg-GRKInh; **, P<0.01 vs. Tg-FASN; ***, P<0.001 vs. Tg-FASN).

The decreased lipid load of Tg-GRKInh/FASN hearts was accompanied by a significantly decreased cardiac expression of the acetyl-CoA carboxylase alpha (Acaca), which mediates an essential step of fatty acid synthesis by catalysing the conversion of acetyl-CoA into malonyl-CoA (Fig. 19). Notably, GRKInh largely prevented the up-regulation of Acaca, i.e., a gene up-regulated by hypoxia (706), which was commonly triggered at the onset of heart failure induced by Tg-FASN, pressure overload and Pparg (Fig. 19, cf. Fig. 6 and Fig. 8).

![Figure 19](image)

**Figure 19** - Cardiac Acaca expression in Tg-FASN, double-transgenic Tg-GRKInh/FASN, B6 and Tg-GRKInh mice (±SD n=5; ***, P<0.001 vs. Tg-GRKInh/FASN, B6 and Tg-GRKInh hearts).

Although the expression of the human FASN transgene was not significantly altered between single-transgenic Tg-FASN and double-transgenic Tg-GRKInh/FASN hearts (Fig. 20A, left panel), GRKInh led to a significantly decreased expression of the endogenous murine Fasn gene, which is also a hypoxia-induced Pparg target (707), and shows up-regulated expression...
in Tg-\textit{FASN} hearts with signs of heart failure (Fig. 20\textit{A}, right panel, and cf. Fig. 6, upper panel). Concomitantly, the total cardiac FASN/Fasn protein level of Tg-GRKInh/\textit{FASN} hearts was significantly decreased relative to that in Tg-\textit{FASN} hearts (Fig. 20\textit{B}).

Together these experiments show that GRK2 inhibition retards the \textit{FASN}-induced dysfunction of the cardiac substrate metabolism and lipid overload. Concomitantly with the decreased lipid load, the development of signs of heart failure such as cardiac dysfunction, cardiac hypertrophy, cardiac ATP depletion and \textit{FASN}/\textit{Fasn}-mediated cell death, was significantly retarded (Fig. 21 and 22).

\textbf{Figure 20} - (\textit{A}) Cardiac expression of transgenic \textit{FASN} (left panel) (±SD, \textit{n}=5; ***, \textit{P}<0.001 vs. B6), and expression of the endogenously expressed murine \textit{Fasn} (right panel) (±SD; \textit{n}=5; ***, \textit{P}<0.001 vs. Tg-GRKInh/\textit{FASN} and B6). (\textit{B}) Immunoblot detection of the FASN/Fasn protein in cardiac tissue extracts from Tg-\textit{FASN} relative to Tg-GRKInh/\textit{FASN} mice (±SD; \textit{n}=5 hearts/group; **, \textit{P}=0.0016).

\textbf{Figure 21} - (\textit{A}-\textit{C}), The left ventricular ejection fraction (\textit{A}), the heart-weight-to-body-weight ratio (\textit{B}), and the cardiac ATP content (\textit{C}) of Tg-\textit{FASN} mice relative to double-transgenic Tg-GRKInh/\textit{FASN} mice and age-matched B6 controls (±SD, \textit{n}=5; *, \textit{P}<0.05, **, \textit{P}<0.01 and ***, \textit{P}<0.001 vs. Tg-\textit{FASN})
5.3.4.9 GRK2 inhibition promotes ERK axis-dependent inhibition of Pparg transcriptional activity

We investigated the mechanism that accounts for GRK2 inhibition-mediated protection against FASN-induced cardiolipotoxicity, and focused on the interrelationship between GRK2 inhibition-mediated ERK axis activation and the inactivation of Pparg. Several lines of evidence support such a relationship. (i) The expression of several heart failure-related Pparg targets such as adiponectin (Adipoq), resistin (Retn) and uncoupling protein 1 (Ucp1), is down-regulated by ERK-dependent inactivation of Pparg, partially by involving serine-273 phosphorylation (704) (708). (ii) GRK2 inhibition enhances the activation of the ERK cascade (512) (709). (iii) Additionally, the reversal of palmitate toxicity can be achieved by ERK activation, e.g., triggered by AMPK signaling (685), i.e., the signaling pathway that was re-sensitised by GRK2 inhibition (cf. Fig. 17). Conversely, excess palmitate down-regulated the ERK axis (685).

In agreement with palmitate-mediated inhibition of ERK (685), the cardiac content of activated phospho-ERK1/2 was low in Tg-FASN hearts relative to double-transgenic Tg-GRKInh/FASN hearts (Fig. 23).

**Figure 22** - The number of TUNEL-positive cardiomyocytes (±SD, n=5; ***P<0.001 vs. Tg-FASN). The right panel shows representative immunohistological sections of TUNEL staining (bar: 20 μm).

**Figure 23** - (A) Immunohistological detection of activated phospho-ERK1/2 in cardiac sections of a Tg-FASN mouse relative to a Tg-GRKInh/FASN mouse (bar: 40 μm). Histology experiments are representative of four different mice/group. (B) Immunoblot detection of activated phospho-ERK1/2 (upper panel) and total ERK1/2 (lower panel) in cardiac tissue extracts from Tg-FASN relative to Tg-GRKInh/FASN mice (n=4 hearts/group).
Notably, GRKInh enhanced the activation of ERK2 (Fig. 23B), which is cardioprotective and protects the myocardium against ischemic injury (710). Concomitantly with enhanced ERK1/2 activation, serine-273 phosphorylated Pparg was increased in double-transgenic Tg-GRKInh/FASN hearts relative to Tg-FASN hearts (Fig. 24).

In agreement with the ERK-dependent inactivation of Pparg (704) (708) (711), the enhanced phosphorylation of Pparg on serine-273 and serine-112 of double-transgenic Tg-GRKInh/FASN cardiomyocytes was accompanied by a significantly decreased Pparg transcription factor DNA-binding activity compared to that of Tg-FASN cardiomyocytes (Fig. 25). The decreased Pparg activity of Tg-GRKInh/FASN cardiomyocytes was dependent on an activated ERK axis because the MEK inhibitor PD0325901 blunted the phosphorylation of Pparg on serine-273 and serine-112, and led to a significant up-regulation of the Pparg transcriptional activity of Tg-GRKInh/FASN cardiomyocytes (Fig. 25).

**Figure 24** - Immunoblot detection of pS273-Pparg (upper panel) and total Pparg (lower panel) in cardiac tissue extracts from Tg-FASN and Tg-GRKInh/FASN mice (n=4 hearts/group).

**Figure 25** - Left panel: Immunoblot detection of pS273-Pparg (upper panel), pS112-Pparg (middle panel), and Pparg (lower panel), respectively. Right panel: Pparg activity of nuclear extracts from Tg-FASN relative to Tg-GRKInh/FASN cardiomyocytes (3F protocol) treated without (-) or with (+) the MEK inhibitor PD0325901 (0.5 μM) (±SD; n=4; ***,P<0.001).
Concomitantly with *Pparg* inhibition, the expression of ERK-regulated, heart failure-related *Pparg* targets (i.e., *Ucp1, Adipoq*) was significantly lower in double-transgenic Tg-GRKInh/FASN hearts compared to Tg-FASN hearts (Fig. 26). Concordantly with decreased signs of heart failure, GRKInh also led to a significantly decreased expression of the heart failure marker and *Pparg* target gene, resistin (*Retn*) (Fig. 26).

![Figure 26](image)

*Figure 26* - Cardiac expression of heart failure-related *Pparg* target genes in Tg-FASN, double-transgenic Tg-GRKInh/FASN, and B6 control mice (±SD; n=4; ***, *P*<0.001 vs. Tg-GRKInh/FASN and B6).

Because *Adipoq* and *Retn* are heart failure-related *Pparg* targets (712) (713) that are induced by serine-273 phosphorylation-deficient *PPARG*-S273A (708), these data are compatible with the notion that cardioprotective GRK2 inhibition could involve the suppression of *Pparg*-dependent cardiolipotoxic gene expression by enhanced ERK-mediated serine-273 phosphorylation and the inactivation of *Pparg*.

### 5.3.4.10 Inhibition of Fasn lowers the cardiolipotoxicity induced by serine-273 phosphorylation-deficient PPARG-S273A

We analysed the impact of phosphorylation-deficient *PPARG*-S273A on cardiomyocyte function. Our experiments showed that both cardiomyocyte FFA content and the Fasn protein were triggered by *PPARG* activated with the *PPARG* agonist rosiglitazone and by *PPARG*-S273A (Fig 27A, C, D). These data are in agreement with those from previous studies, which have shown that *PPARG* serine-273 dephosphorylation can enhance the expression of Fasn (714).

Concomitantly with the FFA load, cardiomyocyte dysfunction developed as evidenced by a significantly decreased cardiomyocyte ATP content induced either by rosiglitazone-activated *PPARG* or *PPARG*-S273A, respectively (Fig. 27B). The inhibition of *Fasn* by RNAi (Fig. 27C, D) led to significantly decreased cardiomyocyte FFA content and largely prevented the decrease in cardiomyocyte ATP (Fig. 27A, B). Together these findings provide evidence that *PPARG*-S273A deteriorates cardiomyocyte function by regulating *Fasn*. 
Figure 27 - Inhibition of Fasn decreases cardiolipotoxicity induced by serine-273 phosphorylation-deficient PPARG-S273A. (A, B) Neonatal rat cardiomyocytes were transfected with PPARG or PPARG-S273A mutant, and incubated in the absence (-) or presence (+) of rosiglitazone (Rosiglit., 5 μM), and Fasn was inhibited by RNAi (siFasn) as indicated. The free fatty acid (A) and ATP (B) contents of cardiomyocytes were determined [±SD; n=4; *, P<0.05; **, P<0.01; ***. P<0.001 vs. controls transfected with stealth control RNAi (-); Dunnett’s multiple comparison test]. (C) Immunoblot detection of Fasn (upper panel) and PPARG (middle panel) in cardiomyocyte lysates. The lower panel is a control immunoblot detecting Gnb. (D) Immunofluorescence localization of Fasn (red) and the transmembrane-spanning AT1 receptor (AT1R; green) in cardiomyocytes without (Control) or with transfection of PPARG, PPARG-S273A, siFasn and treatment with rosiglitazone (Rosiglit.) as indicated. Fasn was detected with affinity-purified rabbit anti-Fasn antibodies followed by F(ab)2 fragments of Alexa Fluor 546-labelled secondary antibodies, and AT1R was detected with affinity-purified rat anti-AT1R antibodies followed by F(ab)2 fragments of Alexa Fluor 488-labelled secondary antibodies. Cell nuclei were stained with DAPI (blue; bar: 20 μm). The immunofluorescence data are representative of four independent experiments.
5.3.4.11 GRK2 inhibition retards the up-regulation of the heart failure-related Ucp1 and mitochondrial uncoupling

In search of additional heart failure-promoting ERK-controlled \textit{Pparg} target genes, we focused on \textit{Ucp1} (704) (715), which exerts mitochondrial uncoupling, a major metabolic feature of the failing heart metabolism (619). GRK2 inhibition by GRKInh led to a decreased cardiac \textit{Ucp1} expression and protein level in Tg-GRKInh/\textit{FASN} mice relative to Tg-\textit{FASN} mice (Fig. 28A and cf. Fig. 26). Moreover, cardiomyocytes from Tg-GRKInh/\textit{FASN} mice showed a significantly decreased oligomycin-insensitive OCR (a measure of mitochondrial uncoupling) compared to Tg-\textit{FASN} cardiomyocytes (Fig. 28B). Conversely, the inhibition of the ERK axis in Tg-GRKInh/\textit{FASN} cardiomyocytes significantly increased the \textit{Ucp1} protein and mitochondrial uncoupling (Fig. 28B,C). These findings indicate that GRK2 inhibition decreased \textit{Ucp1}-dependent mitochondrial uncoupling in Tg-GRKInh/\textit{FASN} cardiomyocytes by enhanced activation of the ERK axis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure28}
\caption{GRK2 inhibition retards up-regulation of heart failure-related \textit{Ucp1} and mitochondrial uncoupling. (A) Immunoblot detection of \textit{Ucp1} protein in heart tissue extracts of Tg-\textit{FASN} relative to Tg-GRKInh/\textit{FASN} mice (n=4 hearts/group). The lower panel shows a control immunoblot detecting Gnb. (B) Increased oligomycin-insensitive OCR of Tg-\textit{FASN} cardiomyocytes relative to Tg-GRKInh/\textit{FASN} cardiomyocytes (3F) treated for 72 h without (-) or with (+) the MEK inhibitor PD0325901 [±SD; n=4; *, \textit{P}<0.05 vs. TgGRKInh/\textit{FASN}(-); ***, \textit{P}<0.001 vs. TgFASN]. Baseline OCR (3F) of Tg-\textit{FASN}, Tg-GRKInh/\textit{FASN} and Tg-GRKInh/\textit{FASN} (+PD) was 13.68±0.98, 22.73±2.18 and 18.09±2.62 pmol/min per µg protein, respectively (±s.d.; n=4). (C) Immunoblot detection of \textit{Ucp1} protein of Tg-\textit{FASN} cardiomyocytes relative to Tg-GRKInh/\textit{FASN} cardiomyocytes treated for 72 h without (-) or with (+) PD0325901 [±SD; n=4; *, \textit{P}<0.05 vs. Tg-GRKInh/\textit{FASN} (-); ***, \textit{P}<0.001 vs. Tg-\textit{FASN}]. The right panels show a representative immunoblot experiment.}
\end{figure}

5.3.4.12 Transgenic Tg-\textit{UCP1} mice developed signs of heart failure and increased mitochondrial uncoupling

To analyse whether an increased cardiac \textit{UCP1} level contributed to heart failure pathogenesis in vivo, we generated Tg-\textit{UCP1} mice with myocardium-specific \textit{UCP1} expression (Fig. 29A, B). Immunoblot detection confirmed the increased cardiac UCP1 protein in Tg-\textit{UCP1} (Tg-6) mice relative to non-transgenic B6 controls (Fig. 29C). In addition to the Tg-6 line, we also used Tg-11 offspring with lower UCP1 protein levels (Fig. 29D).
Aged Tg-UCP1 mice from the Tg-6 line developed cardiac dysfunction with a significantly decreased left ventricular ejection fraction and decreased cardiac ATP content compared to non-transgenic B6 mice whereas cardiac function parameters of the low-expressing Tg-11 line were not significantly different from B6 controls (Fig. 30).

**Figure 29** - Transgenic Tg-UCP1 mice developed signs of heart failure and mitochondrial uncoupling. (A), The transgenic vector used for the generation of Tg-UCP1 mice with myocardium-specific UCP1 expression. (B) PCR identification of founder mice with stable integration of the UCP1 transgene into the genomic DNA. (C, D) Immunoblots show the detection of UCP1 protein in 6 month-old Tg-UCP1 hearts (Tg-6) relative to age-matched B6 hearts (C), and Tg-11 hearts (D), respectively (n=4 hearts/group; the last lane of panel (D) is an additional B6 control). The lower panels show control immunoblots detecting Gnb.

Aged Tg-UCP1 mice from the Tg-6 line developed cardiac dysfunction with a significantly decreased left ventricular ejection fraction and decreased cardiac ATP content compared to non-transgenic B6 mice whereas cardiac function parameters of the low-expressing Tg-11 line were not significantly different from B6 controls (Fig. 30).

**Figure 30** - Left ventricular ejection fraction (left panel) and cardiac ATP content (right panel) of 6 month-old Tg-UCP1 mice (Tg-6) relative to Tg-11 mice and B6 controls (±SD; n=5; ***, P<0.001 vs. B6 control; Dunnett’s multiple comparison test).
Concomitantly with cardiac dysfunction, Tg-UCP1 mice showed cardiac dilatation and loss of heart muscle, whereas the heart-weight-to-body-weight ratio was not significantly different from that in the B6 controls (Fig 31).

Figure 31 - Left panel. Heart-weight-to-body-weight ratio of Tg-UCP1 relative to B6 mice (±SD; n=5). Right panel. Representative histological section of a 6 month-old Tg-UCP1 heart relative to an age-matched B6 control (bar: 2 mm). Histology experiments are representative of four different mice/group.

Signs of heart failure were accompanied by a significant up-regulation of the cardiac Fasn protein in Tg-UCP1 mice compared to non-transgenic B6 controls (Fig. 32). The up-regulation of the Fasn protein by UCP1 could be a consequence of the impaired cardiac function and insufficient tissue oxygen supply, which could induce Fasn up-regulation because it is a hypoxia-induced gene (707).

Figure 32 - Immunoblot detection of Fasn protein in cardiac tissue extracts from Tg-UCP1 relative to B6 mice (±SD; n=4). The left panel shows a representative immunoblot detection for Fasn.

The ensuing increase in palmitate may enhance mitochondrial uncoupling by the transgenic UCP1 protein. In support of that notion, Tg-UCP1 cardiomyocytes showed a significantly increased oligomycin-insensitive (uncoupled) respiration compared to B6 cardiomyocytes (Fig. 33).
Together these experiments indicate that the UCP1 protein could have a major role in the depressed substrate metabolism of a failing heart because up-regulated UCP1 could account for an enhanced palmitate-triggered uncoupled respiration upon FASN induction. Under those conditions, GRK2 inhibition by GRKInh could confer several modes of cardioprotection: (i) By counteracting mitochondrial uncoupling via ERK activation-mediated down-regulation of UCP1 (704); and (ii) by decreasing the FASN-triggered lipid load, which involves, e.g., down-regulation of hypoxia-induced Pparg targets, Acaca and Fasn, and the resensitisation of Prkaa.

5.3.4.13 Inhibition of Ucp1 counteracts PPARG-S273A-induced cardiolipotoxicity

The mechanism of ERK-mediated down-regulation of the Pparg target, Ucp1, is not completely understood (704) (715). Ucp1 is a Pparg target that is highly up-regulated in Tg-FASN and pressure overload-induced heart failure models, and by direct Pparg activation with rosiglitazone (cf. Fig. 6, 7 and 8). Low ERK axis activity in these models could mediate stabilization of transcriptional cofactors that are necessary for Ucp1 induction, i.e., PGC1a or PRDM16 (704). Moreover, recent data have shown that PPARG-S273 dephosphorylation can also enhance the recruitment of PGC1a as a transcriptional cofactor involved in Ucp1 induction (714). To analyse the interplay between Ucp1 and PPARG-S273A-mediated cardiolipotoxicity, we inhibited Ucp1 by RNAi. The inhibition of Ucp1 led to an increased ATP content of cardiomyocytes with rosiglitazone-activated PPARG and with PPARG-S273A expression, while concomitantly decreasing the cardiomyocyte FFA content (Fig. 34).

**Figure 33** - Increased oligomycin-insensitive OCR of Tg-UCP1 cardiomyocytes relative to B6 cardiomyocytes (±SD; n=4 biological replicates; 3F protocol). The right panel shows baseline OCR values.

**Figure 34** - FFA (left panel) and ATP contents (middle panel) of isolated neonatal cardiomyocytes from B6 mice after transfection without (-) or with (+) PPARG, PPARG-S273A, siUcp1 and treatment with rosiglitazone (+rosiglit.) as indicated [±SD; n=4; *,P<0.05; **, P<0.01; ***, P<0.001 vs. control transfected with stealth control RNAi (-); Dunnett’s multiple comparison test]. The right panels show immunoblot detection of Ucp1 and PPARG.
These findings obtained with Ucp1 are analogous to the data obtained with Ucp2, which showed that (i) the Ucp2 knockout decreased the cellular lipid content of pancreatic islets as a consequence of higher palmitate oxidation (716); and (ii), Ucp1 knockdown attenuated the free fatty acid-induced apoptosis of cardiomyocytes (717); and (iii), conversely, Ucp2 overexpression decreased the cardiomyocyte ATP levels (718). Taken together, the data are compatible with the notion that Ucp1 exerts a detrimental role in cardiolipotoxicity, as triggered, e.g., by Pparg activation with rosiglitazone and/or serine-273 dephosphorylation. However, the inhibition of Ucp1 could counteract such cardiotoxic effects.

5.3.4.14 Cardioprotective GRK2 inhibition requires an intact ERK axis

We further investigated the impact of ERK activation on cardioprotective GRK2 inhibition and applied the dual-specific GRK2 and ERK cascade inhibitor, RKIP (689). RKIP-transgenic mice with myocardium-specific expression of the human RKIP (PEBP1) were previously generated and characterised (512). RKIP-transgenic and GRKInh-transgenic hearts display similar inhibition of GRK2 (512). Similarly, isolated neonatal cardiomyocytes of RKIP-transgenic and GRKInh-transgenic mice showed a comparable enhancement of the isoproterenol-stimulated cAMP response (Fig. 35A). This observation confirmed that the two different GRK2 inhibitors were expressed at equivalent levels with regard to the sensitization of the β-adrenergic receptor response. However, in contrast to GRKInh, transgenic hearts that expressed the dual-specific GRK2/ERK cascade inhibitor, RKIP, were characterised by a significantly decreased phosphorylation of Pparg on serine-273 (Fig. 35B, C), which is ERK dependent (704).

![Figure 35](image)

Figure 35 - Cardioprotective GRK2 inhibition requires an intact ERK axis. (A) Isoproterenol-stimulated (100 nM) cAMP levels of neonatal cardiomyocytes isolated from RKIP-transgenic, GRKInh-transgenic and B6 mice (±SD, n=3; ***,P<0.001 vs. B6). (B) Immunoblot detection of pS273-Pparg (upper panel) and total Pparg (lower panel) in cardiac tissue extracts of RKIP-transgenic mice relative to B6 controls. (C) Quantitative evaluation of immunoblot data (±SD, n=3; ***,P<0.001 and **,P<0.01 vs. RKIP; and *,P<0.05 vs. B6).

We next determined the expression of cardiac Pparg targets of RKIP-transgenic hearts because the inhibition of Pparg serine-273 phosphorylation induces Pparg target gene expression (704). Gene expression analysis revealed the significant up-regulation of heart failure-related Pparg targets (Fig. 36).
Some of those highly up-regulated heart failure genes, such as adipsin (Adn), adiponectin (Adipoq), fatty acid synthase (Fasn), resistin (Retn), and uncoupling protein-1 (Ucp1), are reportedly induced by the inhibition of ERK and/or serine-273 dephosphorylation of Pparg (704) (708) (714), which was triggered by RKIP (cf. Fig. 35B,C). In agreement with heart failure-related adipogenic target gene up-regulation, cardiac lipid load developed, and cardiac dysfunction became evident in hearts of aged RKIP-transgenic mice [Fig. 37A-D; and ref. (707) (708) (709) (710) (711)].

In vitro data documented the dysfunctional cardiomyocyte energetics of RKIP-transgenic cardiomyocytes compared to the normal metabolism of GRKInh-transgenic cardiomyocytes (Fig. 38A-E). Taken together our data strongly suggest that an intact ERK axis is required for GRK2 inhibition-dependent protection against dysfunctional metabolic substrate use.
5.3.4.15 GRK2 inhibition retards the development of heart failure signs, cardiac lipid load and Pparg target gene induction in a pressure overload-induced heart failure model

Thus, we have presented evidence for GRKInh-mediated protection of Tg-FASN hearts. However, up-regulation of the Pparg-dependent lipid metabolic process is also a characteristic feature of heart failure models that imitate major risk factors of patients such as chronic pressure overload (cf. Fig 6, lower panel and cf. Fig. 35-38). We therefore analysed the effect of GRKinh on the cardiac lipid metabolism in a chronic pressure overload-induced heart failure model imposed by long-term (6 months) abdominal aortic constriction, AAC.

Figure 38 – (A, B) Real-time measurement of OCR (A) and ECAR (B) of neonatal cardiomyocytes from RKIP-transgenic mice was performed under basal conditions and after the creation of an adult-like lipogenic milieu by the 3F protocol. Neonatal cardiomyocytes from Tg-GRKInh mice were measured under basal conditions. The relative values normalised to the baseline (upper panels), and the absolute values of OCR and ECAR (lower panels) are presented. (C-E) The Etomoxir-blocked fraction of OCR, which represents fatty acid β-oxidation (C), the 2-deoxyglucose-blocked fraction of ECAR, which represents glycolysis (D), and the ratio of glycolysis/β-oxidation (E) are also given. The data are shown as the means ±SD; n=6 technical replicates (A, B) and n=3 biological replicates (C-E); ***, P<0.001 vs. RKIP (C, E), **, P<0.01 vs. RKIP-3F (D) and RKIP (E) and *, P<0.05 vs. RKIP-3F (D, E).
In agreement with previous data (512), AAC promoted cardiac hypertrophy with dilatation in non-transgenic mice, whereas Tg-GRKInh mice showed a significantly decreased cardiac hypertrophy (Fig. 39).

**Figure 39** - GRK2 inhibition retards the development of heart failure signs, cardiac lipid load and Pparg target gene induction in a pressure overload-induced heart failure model. (A) Representative histological sections of hearts from a 10-month-old B6 mouse (AAC) relative to an age-matched GRKInh-transgenic mouse (AAC+GRKInh) after 6 months of pressure overload imposed by AAC. The lower panels show age-matched sham-operated control hearts, bar: 2 mm. (B, C) The heart-weight-to-body-weight ratio (B), and the left ventricular ejection fraction (C) of 10-month-old GRKInh-transgenic mice with 6 months of AAC (AAC+GRKInh) relative to age-matched non-transgenic B6 mice with 6 months of AAC (AAC). Age-matched sham-operated non-transgenic B6 mice (Sham) and sham-operated GRKInh-transgenic mice (Sham+GRKInh) served as controls. Data are shown as the means ±SD, n=4 (*, P<0.05 vs. AAC+GRKInh; **, P<0.01 vs. AAC; ***, P<0.001 vs. AAC). Histology experiments are representative of four different mice/group (B).

The development of cardiac dysfunction upon AAC, as assessed by the left ventricular ejection fraction, was also significantly retarded in Tg-GRKInh mice (Fig. 39C). In addition to the improved cardiac function, the AAC-stimulated up-regulation of the cardiac Fasn protein was blunted in Tg-GRKInh mice (Fig. 40A).
Concomitantly, oil red O staining of cardiac sections indicated that the AAC-triggered lipid load was lower in Tg-GRKInh mice (Fig. 40B). In agreement with the decreased lipid-induced cardiolipotoxicity, Tg-GRKInh mice showed a significantly decreased number of AAC-induced TUNEL-positive cardiomyocytes compared to the number in non-transgenic mice with AAC (Fig. 41).

**Figure 40** - (A) Immunoblot detection of Fasn in cardiac tissue extracts of 10-month-old B6 mice with 6 months of AAC relative to age-matched GRKInh-transgenic mice with 6 months of AAC (n=4 hearts/group, left blot). Under the experimental conditions, the Fasn protein (lane 1, A, positive control of a 10-month-old B6 heart with 6 months of AAC) was not detectable in cardiac tissue extracts from sham-operated B6 (sham) or GRKInh-transgenic mice (n=2; right blot). The lower panels show control immunoblots detecting Gnb. (B) Oil red O staining of cardiac sections from the different groups of mice (bar: 40 μm). Histology experiments are representative of four different mice/group (B).

**Figure 41** - The number of TUNEL-positive cardiomyocytes (±SD; n=4; ***P<0.001 vs. AAC). The left panel shows representative immunohistological sections of TUNEL staining (bar: 20 μm).
In view of the decreased AAC-induced cardiolipotoxicity, we analysed the potential effect of GRK2 inhibition on Pparg-inhibitory serine-273 phosphorylation. Immunoblot detection indicated an increased cardiac content of serine-273-phosphorylated Pparg of Tg-GRKInh hearts with AAC compared to non-transgenic B6 mice with AAC (Fig. 42).

![Immunoblot detection of pS273-Pparg (upper panels) and total Pparg (lower panels) in cardiac tissue extracts from the different groups of mice [n=4 hearts/group (left panel), and n=2 hearts/group (right panel)].](image)

The increased level of Pparg-inhibitory serine-273 phosphorylation was accompanied by a significantly lower expression of heart failure-related Pparg targets, which are blunted by ERK activation and/or ERK-dependent Pparg serine-273 phosphorylation, i.e., Ucp1, Adipoq and Retn (Fig. 43). Taken together, cardioprotective GRK2 inhibition with GRKInh retarded the up-regulation of heart failure-related and ERK-inhibited Pparg targets, and enhanced Pparg-inhibitory serine-273 phosphorylation.

![Expression of heart failure-related Pparg targets in hearts from 10-month-old B6 mice with 6 months of AAC (AAC) and age-matched Tg-GRKInh mice with 6 months of AAC (AAC+GRKInh) relative to sham-operated controls (±SD; n=4; ***, P<0.001 vs. AAC+GRKInh, Sham, and Sham+GRKInh).](image)

Figure 42 - Immunoblot detection of pS273-Pparg (upper panels) and total Pparg (lower panels) in cardiac tissue extracts from the different groups of mice [n=4 hearts/group (left panel), and n=2 hearts/group (right panel)].

Figure 43 - Expression of heart failure-related Pparg targets in hearts from 10-month-old B6 mice with 6 months of AAC (AAC) and age-matched Tg-GRKInh mice with 6 months of AAC (AAC+GRKInh) relative to sham-operated controls (±SD; n=4; ***, P<0.001 vs. AAC+GRKInh, Sham, and Sham+GRKInh).
5.3.4.16 Down-regulation of endogenous Ucp1 retards the development of cardiac dysfunction in a pressure overload-induced heart failure model

Although previous studies have provided evidence for the involvement of Adipoq and Retn in heart failure pathogenesis of patients and animal models (712) (713) (719) (720) (721), the role of Ucp1 up-regulation in cardiac dysfunction is less clear. Notably, the onset of heart failure signs in different heart failure models was characterised by a strong cardiac Ucp1 up-regulation (cf. Fig. 6-8), and transgenic expression of UCP1 in the heart promoted signs of heart failure (cf. Fig. 29-34). To investigate the effect of Ucp1 up-regulation in the AAC-induced heart failure model, we down-regulated the endogenously expressed Ucp1 by lentiviral transduction of an miRNA that targets Ucp1 by RNA interference (Fig. 44A,B). The down-regulation of Ucp1 in the AAC-induced heart failure model led to a small, but significant retardation of the development of AAC-induced cardiac dysfunction (Fig. 44C). These findings provide further evidence (cf. Fig. 29-34) for the role of Ucp1 up-regulation in AAC-induced signs of heart failure.

Figure 44 - Down-regulation of endogenous Ucp1 retards the development of cardiac dysfunction in a pressure overload-induced heart failure model. (A, B) Endogenous Ucp1 expression (A) and Ucp1 protein level (B) in hearts of B6 mice with 2 months of AAC and transduction of a control lentivirus (AAC+miCont.) or a lentivirus targeting Ucp1 by RNAi (AAC+miUcp1) relative to sham-operated B6 controls. (C) Down-regulation of Ucp1 retarded the AAC-triggered decrease in the left ventricular ejection fraction (AAC+miUCP1) relative to AAC-subjected B6 mice transduced with a control lentivirus (AAC+miCont.). The data are shown as the means ±SD (n=4; *,P<0.05 and ***,P<0.001 vs. Sham B6; Dunnett’s multiple comparison test).

5.3.4.17 Low efficacy of GRKInh in retarding the cardiac phenotype of PPARG-S273A-transgenic mice

Our data provided evidence that GRK2 inhibition counteracts the dysfunctional cardiac substrate use of heart failure (at least partially) by ERK-dependent inactivation of Pparg involving serine-273 phosphorylation. To further analyze the role of PPARG and a serine-273 phosphorylation-deficient PPARG mutant (PPARG-S273A) in the heart, we generated transgenic mice with myocardium-specific expression of wild-type PPARG and mutated PPARG-S273A, respectively (Fig. 45A). Histological analysis revealed that transgenic
PPARG-S273A mice developed cardiac hypertrophy with dilatation, which was already evident in newborn mice (Fig. 45B). The dilatation of the PPARG-S273A-expressing heart was greater than that of the PPARG-expressing heart (Fig. 45B), although the cardiac PPARG protein level was comparable between the two transgenic groups (Fig. 45C).

Next we investigated the effect of GRK2 inhibition by GRKInh, and compared single transgenic mice (PPARG-S273A and PPARG), with double-transgenic PPARG-S273A+GRKInh-expressing and PPARG+GRKInh-expressing mice, respectively. All 4 groups of mice showed similar cardiac PPARG expression (Fig. 46, left panel).

Figure 45 - Low efficacy of GRKInh in retarding the cardiac phenotype of PPARG-S273A-transgenic mice. (A) A transgenic vector used for the generation of transgenic mice with myocardium-specific expression of PPARG (and PPARG-S273A). (B) Cardiac sections from newborn transgenic mice that expressed PPARG-S273A or wild-type PPARG relative to a non-transgenic B6 control. Histological sections were stained with hematoxylin-eosin (H&E), and are representative of three mice/groups. (C) Immunoblot detection of PPARG/Pparg in cardiac tissue extracts of 4-week-old mice with transgenic PPARG-S273A (S273A) expression or PPARG expression (as indicated) relative to non-transgenic B6 controls (n=4 hearts/group). The lower panel is a control immunoblot detecting Gnb.
Despite of having similar PPARG expression, the GRKInh largely prevented the cardiac hypertrophy of PPARG-expressing mice, whereas the effect of GRKInh on PPARG-S273A-expressing mice was not significant (Fig. 46, right panel). In addition, there was an increased postnatal mortality of PPARG-S273A-expressing mice compared to wild-type PPARG-expressing mice (47.8% vs. 9.5%), which was not rescued by the GRK2 inhibitor (Fig. 47).

We determined the expression of selected heart failure-related Pparg targets, i.e., Adipoq, Retn, Fasn and Ucp1. The genes were all significantly up-regulated in the cardiac tissue of four week-old PPARG-S273A-transgenic mice compared to non-transgenic B6 mice (Fig. 48), which confirms the heart failure-like phenotype of newborn PPARG-S273A transgenic mice. We found that the effect of GRKInh was not significant in reducing the PPARG-S273A-mediated up-regulation of Adipoq, Retn, Fasn and Ucp1 (Fig. 48). In contrast to PPARG-S273A-transgenic hearts, the transgenic expression of PPARG caused significantly less up-regulation of selected Pparg targets, and only the expression of cardiac Adipoq and Fasn was significantly increased in four week-old mice (Fig. 48). The up-regulation of Adipoq in PPARG-transgenic mice may have contributed to the cardiac hypertrophy (cf. Fig. 45B and 46, right panel) because Adipoq is required for pro-hypertrophic signaling during pressure overload (712).
**Figure 48** - Gene expression analysis of heart failure-related *Pparg* targets in hearts from 4-week-old transgenic mice that express *PPARG-S273, PPARG-S273+GRKInh, PPARG, PPARG+GRKInh* relative to non-transgenic B6 hearts (±SD, n=4, *, *P*<0.05 and ***, *P*<0.001 vs. B6 control, Dunnett’s multiple comparison test).

**Figure 49** - The cardiomyocyte energetics was determined with neonatal cardiomyocytes isolated from transgenic mice with myocardium-specific expression of *PPARG-S273A* (S273A), *PPARG-S273A+GRKInh, PPARG, PPARG+GRKInh* and non-transgenic B6 mice. The Etomoxir-blocked fraction of OCR, which represents fatty acid β-oxidation (left panels), the 2-deoxyglucose-blocked fraction of ECAR, which represents glycolysis (middle panels), and the ratio of glycolysis/β-oxidation (right panels) were determined under basal conditions (upper panels) and after the creation of an adult-like lipogenic milieu by the 3F protocol for 10 days (lower panels). The data are shown as the means ±SD; n=3 biological replicates; *, *P*<0.05, **, *P*<0.01 and ***, *P*<0.001 vs. B6; Dunnett’s multiple comparison test).
In agreement with the heart failure-like phenotype of PPARG-S273A-expressing mice, the cardiomyocyte energetics of neonatal cardiomyocytes from PPARG-S273A-transgenic mice showed an overall depressed substrate metabolism under basal conditions with predominant glycolysis. This heart failure-like substrate use was not rescued by co-expression of GRKInh (Fig. 49). Conversely, PPARG-expressing cardiomyocytes showed predominant β-oxidation under basal conditions indicative of lipid load, which was retarded by GRKInh (Fig. 49). Upon induction of an adult-like metabolism by the 3F protocol, PPARG-expressing cardiomyocytes shifted to a heart failure-like metabolic substrate use (Fig. 49). In contrast to PPARG-S273A, the development of the PPARG-induced heart failure-like phenotype was retarded by GRKInh (Fig. 49). Together these findings provide evidence for an enhanced cardiac deterioration of PPARG-S273A-transgenic mice compared to PPARG-WT mice. Moreover, GRKInh was inefficient in retarding the development of the PPARG-S273A-induced cardiometabolic dysfunction and the up-regulation of PPARG-S273A-regulated targets.

5.3.5. Discussion

In the present study, we investigated whether GRK2 inhibition could be a specific approach for targeting of the dysfunctional cardiac substrate metabolism, which is characteristic of late-stage heart failure (677) (678) (679). To reproduce the dysfunctional cardiac substrate use, we generated a novel transgenic model with myocardium-specific FASN expression. The model imitated the up-regulation of FASN, which occurs in patients with heart failure (674) (682). In the context of cardiovascular disease and heart failure, the up-regulation of FASN could be a direct consequence of decreased cardiac output and insufficient oxygen supply because FASN is a hypoxia-induced gene (707). Because cardiac ischemia is triggered by major cardiovascular risk factors such as pressure overload and atherosclerosis, up-regulation of FASN could also be an early and causative event in the pathogenesis of heart failure. In agreement with this notion, we found that the sole expression of FASN was sufficient to trigger the signs of heart failure. Additionally, models of heart failure, which imitated cardiovascular risk factors of patients such as chronic pressure overload or advanced atherosclerosis, also showed up-regulation of cardiac Fasn (674).

How could FASN advance the symptoms of heart failure? Initially, up-regulation of FASN might be considered to be beneficial by supplying more energy substrate to the heart muscle. However, in the long-term, the uncontrolled accumulation of palmitate as the major lipid synthesised by FASN could activate the heart failure-promoting transcription factor Pparg, as has been documented by up-regulation of the Pparg-dependent lipid metabolic process in Tg-FASN hearts and various other models of heart failure. Because Fasn is also a Pparg target, a vicious cycle of FASN/Fasn-induced Fasn could be triggered, which finally results in cardiotoxic lipid load, dysfunctional substrate use, and mitochondrial uncoupling due to palmitate-triggered activation of Ucp1 (Fig. 50). The accumulation of palmitate further promotes pro-apoptotic signaling and inhibits the pro-survival ERK axis (16). As a result, there is an enhanced expression of heart failure-associated Pparg targets that are triggered by ERK inhibition (704) (708), such as Adipoq (712) (719) (720), Retn (713) (721), and Ucp1. Ensuing cardiomyocyte death and remodelling, and impaired cardiac energy generation due to mitochondrial uncoupling could further aggravate the symptoms of heart failure (Fig. 50).
When we applied the Tg-FASN mice as a model of a dysfunctional cardiac substrate metabolism and an additional pressure overload-induced heart failure model, we found that GRK2 inhibition directly interfered with the cardiac lipid accumulation and mediated a reduction in the cardiac Fasn protein. These activities could be attributed (at least partially) to several mechanisms: (i) the inhibition of the endogenous Fasn up-regulation, a hypoxia-induced Pparg target (707), (ii) an interference with fatty acid synthesis by preventing Acaca up-regulation, which is also a Pparg-regulated gene induced by hypoxia (706), and (iii) the enhancement of the fatty acid metabolism by re-sensitisation of Adipor1 and Prkaa-mediated signaling (683). As a consequence, GRK2 inhibition retarded the development of the dysfunctional cardiac substrate use characteristic of late-stage heart failure (Fig. 50).

Cardioprotective GRK2 inhibition required an intact ERK axis to preserve the cardiac energetics because RKIP as a dual-specific GRK2 and ERK cascade inhibitor promoted dysfunction of cardiomyocyte energetics, cardiac lipid load and signs of heart failure. Concomitantly, inhibition of the ERK cascade by human RKIP was accompanied by decreased ERK-dependent phosphorylation of Pparg. A decreased ERK-dependent phosphorylation of Pparg on serine-273 and serine-112 is known to enhance Pparg activity and/or increase Pparg target gene induction (704) (708) (711). Similarly, heart failure-related Pparg targets are triggered by RKIP, resulting in development of cardiac lipid load and cardiac dysfunction (512).

Conversely, GRK2 inhibition by GRKInh led to an increased ERK activation and enhanced the ERK-mediated phosphorylation of Pparg on serine-273. ERK axis activation could be part of the cardioprotective gene expression program initiated by GRK2 inhibition (512) (656) (709). Concomitantly, the expression of heart failure-promoting Pparg targets was blunted, and the appearance of the dysfunctional cardiac substrate metabolism was retarded. In agreement with a causal role of PPARG-S273 phosphorylation in GRKInh-mediated cardioprotection, the
phosphorylation-deficient PPARG-S273A mutant promoted dysfunction of the cardiomyocyte substrate metabolism and caused enhanced postnatal death, which was largely insensitive to GRKInh. In contrast, the phenotype of wild-type PPARG was less severe and could be (at least partially) rescued by GRK2 inhibition. Together these data indicate that the ERK axis may specifically counteract the heart failure-promoting transcription factor Pparg by preventing heart failure-related Pparg target gene induction (704) (722) and/or could confer protection against palmitate-induced endoplasmic reticulum stress (723).

Several heart failure-related Pparg targets are inhibited by ERK-dependent Pparg inactivation (704) (708) with GRKInh, such as Adipoq (712) (719) (720) and Retn (713) (721). By generating Tg-UCP1 mice with myocardium-specific UCP1 expression, our study identified UCP1 as a heart failure-related ERK-regulated Pparg target (704) (715), which was also downregulated upon GRK2 inhibition (Fig. 50). Consequently, GRK2 inhibition could decrease excessive mitochondrial uncoupling as a key event that contributes to inefficient cardiac ATP generation and lipid-induced cardiomyocyte death in heart failure (717) (718).

Although the study was performed with experimental mouse models, the data could also be relevant for the human disease because FASN up-regulation is a characteristic feature of patients with heart failure (674) (682). Because PPARG up-regulation occurs in heart failure patients with pressure-overloaded heart and metabolic syndrome (724), GRK2 inhibition is expected to disrupt a vicious FASN/PPARG cycle in patients who suffer from multiple risk factors (Fig. 50). Such a situation was modelled with rosiglitazone-treated ApoE-/- mice because these mice are prone to the development of atherosclerosis and insulin resistance (725), and thereby mimic the risk profile of patients with enhanced PPARG activation and cardiovascular disease. In this model, Pparg activation triggered the up-regulation of Fasn and signs of heart failure within two months. The causal interplay between Fasn and Pparg-induced cardio-toxicity was demonstrated by RNAi-mediated inhibition of Fasn, which prevented the Pparg-induced cardio-lipotoxicity and signs of heart failure. Because GRK2 inhibition also mediated the down-regulation of FASN-dependent cardio-lipotoxicity, patients with high morbidity and multiple risk factors may benefit from GRK2 inhibition. The additional insulin sensitivity-enhancing activity of GRK2 inhibition (651) (656) may further increase the value of such a strategy.

Moreover, heart-specific GRK2 inhibition could become a cardioprotective combination partner for a novel class of insulin sensitivity-enhancing PPARG activators, which rely on the inhibition of ERK-dependent PPARG phosphorylation for antidiabetic activity (704), but have promoted signs of heart failure in clinical trials (726).

In sum, our study provides strong evidence that cardioprotective GRK2 inhibition specifically targets the dysfunctional cardiac substrate use that is a symptom of late-stage heart failure (Fig. 50). The identified targeting approach could stimulate the development of new therapeutic strategies.
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Conflict of interest: The authors declare that they have no conflict of interest with the content of this article.

Author contributions: J.A., M.G. and U.Q. analysed the data and produced all of the figures. J.A. and X.F. generated the transgenic mice. J.A. and U.Q. wrote the main manuscript text. All of the authors reviewed the results and approved the final version of the manuscript.

Footnotes
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The abbreviations used are: AAC, abdominal aortic constriction; Acaca, acetyl-CoA carboxylase alpha; Adipoq, adiponectin; Adipor1, adiponectin receptor protein 1; Arrb1, beta-arrestin-1; B6 mice, C57BL/6J mice; BrdU, 5-bromo-2’-deoxyuridine; CPT-1, carnitine palmitoyl transferase 1; DAG, diacylglycerol; 2-DG, 2-deoxyglucose; ECAR, extracellular acidification rate; ERK, extracellular signal-regulated kinase; FASN, fatty acid synthase; FFA, free fatty acids; GO, gene ontology; GRK2, G-protein-coupled receptor kinase-2; GRKin, GRK2-specific peptide inhibitor with the peptide sequence MAKFERLQTVTNYFITSE; IBMX, 3-isobutylmethylxanthine; MAPK, mitogen-activated protein kinase; α-MHC, α-myosin heavy chain; MEM, minimum essential medium; OCR, oxygen consumption rate; PPARG, peroxisome proliferator-activated receptor γ; Prkaa1/2, protein kinase, AMP-activated alpha 1/2 catalytic subunit; Retn, resistin; RKIP, raf kinase inhibitor protein; Tg-FASN mice, transgenic mice with myocardium-specific expression of FASN; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; UCP1, uncoupling protein 1.
6.1 Discussion of thesis project-1 “Microarray gene expression profiling reveals antioxidant-like effects of angiotensin II inhibition in atherosclerosis”

In the first part of my thesis, I investigated the effect of antioxidant treatment on the development of atherosclerosis in ApoE-deficient mice, which is an established transgenic disease model. The antioxidant treatment approach is relevant in view of the documented role of oxidative stress as a risk factor for atherosclerosis progression. The research project compared the effects of two compounds, i.e. the ACE inhibitor captopril and the antioxidant vitamin E. These compounds were chosen in view of the fact that captopril and vitamin E share overlapping antioxidant mechanisms. Both tested compounds, vitamin E and the ACE inhibitor captopril, reduce the activity of NADPH oxidase, which is the main producer of superoxide (O$_2^-$), an important reactive oxygen species (ROS) involved in the generation of atherosclerosis.

It is well established that vitamin E is a free radical scavenger, which scavenges perhydroxyl radicals and thereby acts directly as an antioxidant (727). In addition to its direct antioxidant capacity, vitamin E could decrease the activity of NADPH oxidases by inhibition of PKC alpha (728). ACE inhibition with captopril also decreases the activity of NADPH oxidases, which is partially attributed to a blunted angiotensin II-mediated AT1 receptor stimulation of NAPDH oxidases (729). In addition, the sulfhydryl (SH) group-containing captopril has a direct antioxidant effect in vitro, which distinguishes captopril from other ACE inhibitors such as enalapril (730).

**Effects of vitamin E on atherosclerosis**

The atherosclerosis-lowering activity and/or interference with cardiovascular risk factors of vitamin E has been proven in mice, rats, rabbits and humans by several studies investigating its effect on markers of oxidative stress and its effect on the arteries (731). Several studies were conducted with animals and humans prone for the development of cardiovascular diseases. Complementary to my study, a study by Pratico et al found that vitamin E retarded the progression of atherosclerosis in ApoE-deficient mice (503). This group focused on the direct antioxidant effect of vitamin E and showed that vitamin E reduced lipid peroxidation and the free radical-stimulated isoprostane generation in the atherosclerosis-prone aorta (503). Another study investigated the effect of oral vitamin E uptake on ApoE-deficient mice subjected to one month of continuous intravenous infusion of angiotensin II. Notably, this study showed that vitamin E had the capacity to directly interfere with angiotensin II-stimulated effects, i.e. the angiotensin II AT1R-stimulated formation of aortic aneurysms (505). In the latter study, vitamin E was also able to reduce oxidative stress within the aorta and attenuated aortic macrophage infiltration (505).

In addition to the direct atherosclerosis-lowering activity, several studies analyzed vitamin E effects on markers of oxidative stress in experimental models of cardiovascular disease. The effects of high-dose treatment of stroke-prone spontaneously hypertensive (SHRSP) rats with vitamin C and vitamin E were compared with non-treated controls to determine the effects of the two antioxidants on hypertension, vascular oxidative stress and vascular responsiveness (728). Although all rats were fed a high-salt diet to enhance blood pressure increase and oxidative stress, the blood pressure of rats treated with vitamin C or vitamin E increased less than the blood pressure of control SHRSP rats (728). Treatment with an antioxidant was also able to reduce the activity of NADPH oxidase which is one of the main sources of O$_2^-$. This was reflected by lower amounts of detectable O$_2^-$ radicals in the arteries derived from rats treated with an antioxidant. Vitamin E and vitamin C protected the superoxide dismutase (SOD)
from downregulation in comparison to non-treated animals, improving the vascular antioxidant capacity. In agreement with these findings, measurements revealed a significantly improved plasma total antioxidant status (TAS) in antioxidant-treated rats. Less oxidative stress resulted in an improved responsiveness of the treated arteries towards acetylcholine, which dilated the arteries. At the same time the media-to-lumen ratio was reduced, indicating less media hypertrophy in rats treated with an antioxidant. These results show that antioxidants preserved the functionality of the aorta's endothelium and media, most probably by a reduction of oxidative stress. Both antioxidants, vitamin C and vitamin E, showed similar effects, but the dosage of vitamin E calculated as international units (IU) needed to achieve equivalent effects was 20-fold smaller (728).

The beneficial activity of vitamin E on individuals with increased cardiovascular risk was also documented in humans. In one study, the antioxidant activity of vitamin E was compared with the antioxidant effects of concentrated red grape juice (RGJ) in hemodialysis patients with elevated cardiovascular risk (732). Regular intake of vitamin E or RGJ both reduced oxidative stress in these patients. The activity of ex-vivo NADPH-oxidase derived from neutrophils in the patients' plasma was significantly reduced by vitamin E and RGJ. The levels of oxidized LDL were also lower in patients receiving vitamin E or RGJ. The intake of RGJ showed stronger effects than vitamin E. Interestingly, only combined intake of vitamin E and concentrated RGJ showed additive effects by lowering ICAM-1 and MCP-1 which are involved in monocyte migration into the aorta (732).

Taken together, vitamin E is a chemically well-defined antioxidant with documented antioxidant activity in the vasculature of mice, rats and humans. In addition, it is one of the few antioxidants with multiple data concerning its antioxidant mechanisms. My study confirmed the atherosclerosis-lowering effect of vitamin E in the experimental model of ApoE-deficient mice.

**Atherosclerosis-lowering activity of ACE inhibitors**

A multitude of studies in animals and humans documents the beneficial effects of ACE inhibitors against cardiovascular risk factors, and cardiovascular disease (733) (734) (735) (736) (737). A direct effect of ACE inhibition on the progression of atherosclerosis was specifically shown in ApoE-deficient mice by Hayek et al., (467). This study used the ACE inhibitor captopril and attributed its atherosclerosis-lowering effect to inhibition of plasma LDL oxidation (467).

ACE inhibition is considered to exert its major antioxidant effect via inhibition of angiotensin II generation and subsequently blunted AT1 receptor stimulation, which promotes NADPH oxidase activation. The detailed mechanism how the AT1 receptor activates the activity of NADPH oxidases is still incomplete. The current knowledge indicates that upon activation by PKC phosphorylation, the NADPH oxidase subunit p47phox translocates to the plasma membrane and forms a complex with the activating subunits NoxA1 and Rac, which then produces more ROS (494) (531). I chose the ACE inhibitor captopril for my study because it interferes with the ACE-dependent angiotensin II generation and thereby reduces the activation of AT1R. Consequently, the AT1R-stimulated NADPH activity and ROS generation are decreased. In addition, among different ACE inhibitors, captopril is the only approved ACE inhibitor in Switzerland which shows antioxidant activity in vitro, a property it shares with vitamin E.
Most likely as a consequence of these dual effects, captopril has stronger preventive effects against the generation of atherosclerotic plaques than other ACE inhibitors in vivo. Several studies reported the superiority of captopril regarding the prevention of atherosclerosis when compared to other ACE inhibitors, e.g. enalapril. In a study investigating the effects of different types of ACE inhibitors on development of atherosclerosis only the sulfhydryl-containing angiotensin converting enzyme inhibitors captopril and zofenopril were able to reduce the cumulative lesion area in ageing ApoE-deficient mice, while enalapril had no effect (730).

It is well known that ACE inhibitors and captopril show not only beneficial effects in experimental models of atherosclerosis but also in humans (735) (736) (737). Data on beneficial effects of ACE inhibitors or angiotensin II-AT1 receptor blockers (ARBs) in atherosclerotic patients were summarized in a meta-analysis of 13 randomized large-scale studies with over 80,000 patients (738). This meta-analysis found that ACE inhibitors or ARBs reduced overall mortality and also reduced the cardiovascular composite primary outcome by 11% in patients with an elevated risk for atherosclerosis, independently from their baseline blood pressure. The cardiovascular composite primary outcome included cardiovascular health, non-fatal myocardial infarction and non-fatal stroke. The major result of this study was that the baseline blood pressure had no influence on the drug's efficacy in lowering all-cause mortality or in reducing the cardiovascular composite primary outcome. This meta-analysis provides evidence that ACE inhibitors or ARBs have a beneficial effect in patients at risk for atherosclerosis (738).

In agreement with these animal and patient data, my study confirmed that treatment with the ACE inhibitor captopril led to a significantly decreased atherosclerotic plaque load in the aorta of ApoE-deficient mice.

**Whole genome microarray gene expression analysis revealed anti-atherogenic activities of vitamin E and captopril**

After documentation of the atherosclerosis-retarding activity of vitamin E and captopril, I investigated underlying mechanisms by whole genome microarray gene expression profiling of aortic specimens. The microarray gene expression study was complemented by (immuno-)histological analysis of aortic specimens.

Whole genome microarray gene expression data were evaluated to search for genes concordantly regulated by vitamin E and captopril. The functions of concordantly regulated and differentially regulated gene sets were determined by gene ontology (GO) analysis. Gene expression data evaluation indicated that both compounds preserved the integrity and functionality of the aorta, partially by protecting the aortic intima and partially by protecting the vascular smooth muscle cells of the aortic media.

Together my study showed that vitamin E and captopril share several antioxidant anti-atherosclerotic properties.

**Vitamin E and captopril preserved the integrity of the aortic intima**

Evaluation of whole genome gene expression data of aortic specimens from the different study groups identified concordantly regulated genes between vitamin E and captopril. The GO analysis found that the expression of 14 genes which foster the integrity of the aortic intima
were increased by vitamin E and captopril relative to the untreated ApoE-deficient controls. Concordantly up-regulated genes included the small-proline rich protein 3 (Sprr3) and repetin (Rptn), which are known to stabilize the stratified epithelium (514). The expression of several other genes was increased more than two-fold by vitamin E and captopril in comparison to non-treated ApoE-deficient mice. Notably, vitamin E and captopril treatment increased the expression level of these genes to the levels detected in non-treated B6 control mice. The increased expression of Sprr3 in mice treated with vitamin E or captopril was confirmed on protein level by immunohistological staining of aortic specimens. The up-regulated Sprr3 protein was localized in the intima of aortas from both treatment groups whereas the Sprr3 protein was barely detectable in the intima of aortas from untreated ApoE-deficient control mice. The protein level of Sprr3 in the treated groups was comparable to that of healthy non-transgenic B6 aortas.

My findings of an intima-protective activity of vitamin E extend previous in vitro studies (739). These in vitro studies investigated the effects of vitamin E on human aortic endothelial cells (HAEC) and showed that d-alpha-tocopherol increased the cells’ “resistance to oxidative stress” and “increased cell viability” (739). The same researchers demonstrated that vitamin E could reduce the expression of ICAM-1, VCAM-1 and E-selectin in HAEC upon stimulation with interleukin-1 beta. As a result, adhesion of HAEC to U937 monocyctic cells was decreased if one cell strain had been exposed to vitamin E. The spontaneous production of IL-8 by HAEC could only be prevented by higher concentrations of vitamin E in the cell media. The production of IL-6, IL-8 and MCP-1 were “dose-dependently suppressed by enrichment of cells with vitamin E” (740). In addition to studies with human aortic endothelial cells, vitamin E was also able to reduce generation of ROS and lipid peroxidation by human keratinocytes (HaCaT), after exposure to ultraviolet A irradiation in vitro (741).

The identification of an intima-protecting activity of captopril complements previous data. A study investigated the effects of angiotensin II inhibition on isolated tail arteries from young and old F344 rats (742) and showed that aged arteries had an improved vasodilator response to acetylcholine after ACE inhibition with perindopril. Notably, after application of the ACE inhibitor perindopril, an AT1 receptor antagonist (losartan, valsartan), or the renin-receptor antagonist aliskiren, the aged arteries showed the same responsiveness towards acetylcholine presented by young arteries. The activity of ROS was also lowered in aged arteries by an ACE inhibitor or angiotensin receptor blocker. These effects were attributed to the inhibition of the local intravascular renin-angiotensin system (742).

Another study demonstrated that ACE inhibition of human umbilical vein endothelial cells (HUVEC) protected these cells against cytotoxicity induced by polymorphonuclear neutrophils (PMN) in vitro. Captopril and enalapril were able to reduce the apoptosis of HUVECs after stimulation with TNF-alpha and incubation with PMN. ACE inhibition also reduced the apoptosis of HUVECs after stimulation with lipoxin-A4 (LXA-4) and subsequent incubation with PMN. Captopril showed effects at lower concentrations than enalapril. The expression of TNF-alpha type 1 receptors on HUVECs was reduced by ACE inhibition, which is of importance as TNF-alpha can induce ICAM-1, VCAM-1 and E-selectin on endothelial cells. In this experiment, only ICAM-1 expression was significantly reduced by ACE inhibition. Captopril and enalapril were able to prevent cytotoxic effects of PMN on HUVECS stimulated with TNF-alpha or LXA-4 (743).
Altogether, vitamin E and ACE inhibitors exert positive effects on endothelial cells which were reflected by the up-regulation of genes coding for proteins involved in integrity of epithelial cells, which share several functions with endothelial cells. The intima-protective activity of captopril could be attributed to its antioxidant properties.

**Vitamin E and captopril promoted aortic media and smooth muscle cell integrity**

Atherosclerosis does not only harm the aortic intima but also the aortic media, which is responsible for the regulation of the luminal diameter and the aorta's elasticity. The analysis of the whole genome gene expression data of the aortic tissue showed a down-regulation of genes responsible for stiffening of the media and showed a decreased expression of genes involved in a dysfunctional lipid metabolism of the aorta. The largest cell population of the media are vascular smooth muscle cells and many genes down-regulated by vitamin E and captopril pointed to an improved functioning of smooth muscle cells in comparison to non-treated ApoE-deficient mice. One of the genes significantly down-regulated by vitamin E and captopril was phospholamban (Pln). Vitamin E and captopril were able to lower the expression of Pln and other harmful genes by ApoE-deficient mice towards expression levels measured in untreated non-transgenic B6 control mice. Immunohistological detection of aortic specimens confirmed this finding and showed less phospholamban in the aortas of ApoE-deficient mice taking vitamin E or captopril in comparison to non-treated ApoE-deficient mice. In addition, immunohistology confirmed that vitamin E and captopril where able to "reduce" the protein expression of phospholamban in the aorta of ApoE-deficient mice to levels measured in non-transgenic B6 mice. The phospholamban was localized in proliferating aortic smooth muscle cells of ApoE-deficient aortas whereas treated aortas did not show a proliferative phenotype of aortic smooth muscle cells. Vitamin E and captopril lowered the expression level of aortic media genes to a similar extent. In contrast, expression levels of genes involved in aortic lipid metabolism were lowered more efficiently by captopril, partially even below gene expression levels measured in control B6 mice.

The findings of my study on the media-protective activity of vitamin E complement and extend many previous studies. In a study conducted by Boscoboinik et al., vitamin E reduced the proliferation of vascular smooth muscle cells in vitro, while other antioxidants showed no effect on the proliferation of VSMCs (744). Further studies showed that vitamin E is able to arrest the cell cycle of rat and human VSMCs at the G1/S transition (745). By inhibition of PKC activity, vitamin E can reduce the activity of NADPH-oxidase, resulting in reduction of the respiratory burst in human macrophages, as a consequence oxidative stress within the aortic tissue is lowered (731). In the glomerulus of diabetic rats, vitamin E activated diacylglycerol kinase, which inactivates diacylglycerol (746). Glucose is known to stimulate PKC activity and results in increased DAG synthesis, a phenomenon counteracted by vitamin E (746). PKC alpha is known to promote cell-survival in epithelial cells, melanoma cells and glioma cells by inhibiting apoptosis (746). Probably, by inhibition of PKC alpha, vitamin E could also reduce the proliferation of VSMC by indirectly inhibiting this mechanism.

Another study showed up-regulation of connective tissue growth factor (CTGF) and fibronectin expression by vitamin E in VSMC in vitro (747). Concerning contractile proteins, supplementation with vitamin E resulted in increased expression of alpha-tropomyosin (748), which is also an indicator that the contractile, healthy phenotype of VSMCs is supported by vitamin E. Tropomyosin interacts with actin to stabilize VSMCs and its loss is involved in phenotype switch towards the proliferative synthetic phenotype of VSMCs (749). The
mechanism of this effect is unknown. Certain type of PKCs can pass into the nucleus, possibly influencing gene transcription (750). The activation of the transcription factor “activator protein 1” (AP-1), which has mainly proliferative effects, is reduced in VSMC after application of vitamin E (751).

The expression of collagenase I/matrix metalloproteinase-1 (MMP-1) by VSMC is also reduced by vitamin E in vitro, probably leading to further reduction of oxidative stress and less degradation of extracellular matrix in vivo (752). A similar observation was made in WWHL rabbits, which showed less MMP-3 expression in their thoracic aorta upon vitamin E intake (753). Furthermore, LDL-oxidation by NADPH-oxidase is reduced by vitamin E, which may limit the inflammatory process in the media (754). By overall reduced generation of ROS and by reduced activity of the lipoxygenase, the generation of immunogenic oxLDL by NADPH-oxidase is reduced, which may reduce inflammation within the media (755).

Beneficial effects of angiotensin II inhibition, e.g. by an ACE inhibitor, on the integrity of the aortic media are well established. Notably, inhibition of the angiotensin II system exerts beneficial effects on vascular smooth muscle cells, which involve the decreased generation of ROS (756). The contribution of angiotensin II-stimulated generation of ROS in the proliferative phenotype of vascular smooth muscle cells was originally found by Griendling et al in 1994. Griendling et al investigated the effects of angiotensin II on VSMCs in vitro and recorded an elevated generation of superoxide anions after incubation of VSMCs with angiotensin II (531). This group was also able to proof that angiotensin II stimulated the activity of NADH and NADPH oxidases in VSMCs, which resulted in an increased generation of ROS (531). Several transcription factors which promote VSMC hypertrophy and proliferation are sensitive to ROS, e.g. AP-1, NF kappa-B and CREB. Daemen and Schwartz showed that “angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall” (757). Angiotensin II-stimulated proliferation of VSMCs involves several mechanisms. Notably, stimulation of the angiotensin II AT1 receptor induces a proliferative phenotype of VSMCs by activation of Gq/11 proteins of heterotrimeric G-proteins (758). By activation of phospholipase(s) C, the Gq/11-stimulated signal transduction cascade generates two intracellular messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). The IP3 binds to and activates the IP3 receptor on the endoplasmic reticulum, which releases Ca2+ from intracellular stores. The ensuing rise in the intracellular free calcium concentration, [Ca2+]i, is an important co-factor for PKC activation. In addition, the second messenger, DAG, is a direct activator of protein kinase(s) C. Angiotensin II-mediated activation of PKC in VSMCs leads to the stimulation of mitogen-activated protein (MAP) kinases and an increased expression of c-fos and c-jun (680) (759). Angiotensin II also stimulates cell growth and proliferation by activation of receptor-coupled tyrosine protein kinases, e.g. the platelet-derived growth factor (PDGF)-receptor and the epidermal growth factor (EGF)-receptor. Non-receptor tyrosine kinases are also activated by the angiotensin II AT1 receptor-stimulated signal transduction cascade, e.g. c-Src, JAK2 and Pyk2 (759) (760) (761). The ensuing phenotype switch of VSMCs from contractile to synthetic/proliferative is a major factor for the pathogenesis of atherosclerosis (762). Again, the effect of the angiotensin II-stimulated signal transduction cascade is synergistic with the generation of ROS (762).

Results of my study complement these previous data and show that inhibition of angiotensin II generation and/or ROS by treatment with the ACE inhibitor captopril or the antioxidant vitamin E, respectively, retarded the proliferative phenotype of VSMCs in the aortic media of ApoE-deficient mice.
Common antioxidant properties, but additional anti-atherogenic effects of the ACE inhibitor captopril

Although captopril and vitamin E exhibited overlapping antioxidant capabilities to booster the expression of protective endothelial genes and to reduce the expression of genes responsible for media stiffness and dysfunctional tissue lipid metabolism, my study found that captopril has additional effects. This conclusion is based on the following findings: (i) macroscopic analysis of the aortas showed that captopril has a stronger reducing effect on development of atherosclerotic plaques than vitamin E. (ii) The stronger atherosclerosis-lowering capacity of captopril was reflected by the microarray gene expression study, which showed that the ACE inhibitor captopril regulated three-fold (3.37-fold) more genes than vitamin E, and most regulated genes were not concordantly regulated between captopril and vitamin E. (iii) The comparison of all regulated probe sets revealed that only captopril was able to reduce the expression of genes involved in the process of leukocyte migration and inflammation. (iv) In addition, the expression of neuronal genes was positively affected by treatment with captopril but not by treatment with vitamin E.

Captopril retarded the aortic recruitment of pro-inflammatory immune cells

Activation of the angiotensin II-AT1 receptor supports local inflammation within the vessel walls which is a hallmark of atherosclerosis. This inflammatory process is initiated by the deposition of cholesterol into the aortic intima and followed by migration of leukocytes, mainly macrophages and lymphocytes, into the aorta's intima and media. Microarray gene expression analysis detected an increased expression of macrophage and T cell marker genes in the atherosclerotic aortas of ApoE-deficient mice, which are two key players in the generation of atherosclerosis (763). My study showed that only the treatment with the ACE inhibitor captopril was able to reduce the number of characteristic macrophage and T cell transcripts to control level.

After migration into the aortic intima or media, monocytes transform into macrophages, which reside in the tissue and cause inflammation. Strongly elevated gene expression of monocyte to macrophage differentiation factor (MMD) was detected in non-treated ApoE-deficient mice. This is of special significance, because the transformation of monocytes into macrophages is followed by phagocytosis of cholesterol derivatives and transformation into foam cells. These cells finally die, release inflammatory cytokines and promote inflammation by debris deposition.

Another gene, which had a massively increased transcription in untreated ApoE-deficient mice, was the chemokine receptor 9 (CCR9). Our previous scientific work had shown that CCR9 is found on macrophages in the atherosclerotic aorta of ApoE-deficient mice (510). The treatment with captopril prevented the upregulation of CCR9 expression in the aorta of ApoE-deficient mice, hinting to a reduced number of macrophages in the aorta. I confirmed this association between a reduced number of transcripts of CCR9 and a decreased number of macrophages in the aorta by immunohistological staining of aortic sections. In contrast to captopril, vitamin E did not lead to a decreased aortic expression of CCR9. Concomitantly, immunohistological analysis documented that vitamin E did not prevent the migration of CCR9-positive macrophages into the atherosclerosis-prone aorta of ApoE-deficient mice.
Taken together, captopril maintained the aortic expression levels of MMD and CCR9 in ApoE-deficient mice at the level of the non-transgenic B6 controls, indicating that migration of monocytes and their transformation into macrophages could be prevented by captopril whereas vitamin E had no significant effect.

Another subset of leukocytes involved in the pathology of atherosclerosis are lymphocytes, especially the T cells as they play an important role for sustaining and promoting inflammation in atherosclerosis. Non-treated ApoE-deficient mice showed a massive upregulation of T cell-specific genes, pointing to an increased migration of CD4+ T helper cells and CD8+ cytotoxic T cells into the vessel walls. The upregulated genes included CD4 and CD8, which are classical surface markers of T helper cells and cytotoxic T cells. In contrast, aortas of ApoE-deficient mice treated with captopril showed a decreased expression of genes coding for CD4 and CD8, indicating a reduced migration of T cells into the vessel wall. Again, vitamin E was not able to retard the aortic recruitment of T cells when compared to untreated ApoE-deficient.

Effects of captopril and ACE inhibition on the migration of pro-inflammatory immune cells into the atherosclerosis-prone aorta are well established (470) (516) (517). However, differential activities between captopril and vitamin E regarding the aortic recruitment of pro-inflammatory immune cells are a major finding of my study and could contribute to the greater anti-atherogenic effects of captopril compared to the weaker effects of vitamin E.

**ACE inhibition with captopril showed neuroprotective effects on perivascular nerves**

Innervation of the vasculature in general and its role in endothelial dysfunction and atherosclerosis is not fully understood. An intact innervation of smooth muscle cells by the autonomic nervous system is important for the regulation of vascular tone and control of trophic functions. Analysis of the microarray of aortic tissue performed by me showed that captopril maintained expression of neuronal genes at the level of healthy B6 controls whereas these genes were strongly downregulated in atherosclerotic ApoE-deficient mice. Immunohistological analysis confirmed the loss of vascular neuron neuropeptide Y synthesis in ApoE-deficient mice by atherosclerosis and this neuronal loss was prevented by captopril.

To my knowledge I was the first to demonstrate protective effects of functional angiotensin II inhibition on vascular neurons in vivo. Several reasons why ACE inhibition exerts protective effects on vascular neurons are conceivable. Atherosclerosis itself is harmful for vascular nerves, because it diminishes blood supply to the neurons and neurites. By preventing the generation of atherosclerotic plaques, and by its capacity to dilate arteries, captopril could improve organ perfusion and oxygen delivery to the vasculature and its nerves. In addition, ACE inhibitors can also have neuroprotective effects which are independent from their effects on blood and oxygen supply. Research studies conducted by our group confirmed neuroprotective effects of ACE inhibition on the central nervous system (763) (764).

**Summary and outlook**

Antioxidant effects and anti-atherosclerotic effects of vitamin E and the ACE inhibitor captopril could be measured in vivo, although effects of vitamin E were more moderate in comparison to ACE inhibition. In contrast to vitamin E, ACE inhibition had additional activities and
decreased the recruitment of pro-inflammatory immune cells into the atherosclerosis-prone aorta.

Another major finding was the protection of vascular neurons by the ACE inhibitor captopril, which was not observed with vitamin E treatment. Captopril is a centrally acting ACE inhibitor, and the herein identified neuroprotective activity in the vascular system could also be relevant for neurodegenerative diseases such as Alzheimer’s disease (AD). In agreement with this notion, the neuroprotective activity of captopril was found in experimental models of familial and sporadic AD (763) (764). Recent clinical data extended the relevance of these experimental studies for patients with neurodegenerative diseases and AD (765) (766) (767) (768). In this context, my whole genome microarray gene expression study could serve to identify additional neuroprotective targets, which are regulated by ACE inhibition, and investigate their potential to be exploited for the development of neuroprotective therapies in the future.
Heart Failure (HF) is a frequent cardiovascular disease with a high mortality. Although many different causes of heart failure are known, the pathomechanisms leading to heart failure are still under investigation. A literature research found conflicting information concerning metabolic changes in the development of heart failure. Therefore, the main goal of my second thesis project was to elucidate the effect of an increased availability of lipids for the heart in vivo. I opted for the generation of transgenic mice with overexpression of the fatty acid synthase (FASN), because the protein level of this enzyme was found to be increased in biopsies from human patients with heart failure (674) (682). In addition, experimental models of heart failure, which mimic major cardiovascular risk factors of patients, i.e. pressure overload and atherosclerosis, also showed an up-regulation of cardiac FASN expression (674). By the generation of FASN-transgenic (Tg-FASN) mice, I wanted to elucidate the effects of an increased availability of lipids on cardiac gene expression, metabolism and cardiac function. A second major aim was to analyze the functional network FASN is part of, how this network is regulated, and how FASN affects cardiac function. This included an analysis of the relationship between FASN and peroxisome proliferator-activated receptor-gamma (PPARG) in the pathogenesis of heart failure. Because FASN-transgenic mice developed signs of heart failure, I aimed at finding a treatment approach to counteract the effects of an increased FASN activity. We chose to analyze the effects of GRK2 inhibition, which is known to exert cardio-protective effects in other experimental models of ischemic cardiovascular disease (536) (769).

A moderately increased cardiac FASN protein promoted signs of heart failure and cardiac lipid overload

To analyze the impact of FASN up-regulation in the heart, I generated FASN-transgenic mice with myocardium-specific overexpression of FASN under control of the alpha-MHC promoter. After the generation of transgenic mice, I first confirmed the cardiac overexpression of FASN on protein level. Immunoblot analysis revealed a ~2.4-fold increased cardiac FASN protein levels in FASN-transgenic mice relative to the non-transgenic B6 controls. This moderately increased FASN protein recapitulates the increase in FASN protein found in cardiac biopsies from patients with heart failure (674). Thus, the newly generated transgenic model was suitable to address the question about the impact of an increased cardiac FASN protein level on heart failure pathogenesis.

Phenotyping of FASN-transgenic mice involved the following steps. To determine the function of hearts with increased FASN protein level, echocardiography was conducted to measure the left ventricular ejection fraction. Phenotypic analysis of the heart included histological analysis of transverse sections of the total heart to determine overall size, diameter of ventricles and diameter of ventricle walls. Increased heart weight-to-body weight ratio was determined as another indicator of cardiac hypertrophy. Visualization of total cardiac lipid content was achieved by staining of cardiac specimens with the lipophilic dye oil red O, while specialized detection methods where used for quantifiable detection of lipid species. To analyze the influence of FASN on cardiac gene expression, I conducted whole genome expression profiling by RNA microarrays. The energy metabolism of isolated neonatal cardiomyocytes was analyzed by measuring levels of glycolysis and beta-oxidation indirectly with a Seahorse extracellular flux analyzer.
Transgenic mice overexpressing FASN showed signs of heart failure at 6 months of age, in contrast to their non-transgenic B6 controls. These signs of heart failure included a significantly reduced left ventricular ejection fraction, an elevated left ventricular inner diameter (LVID) and an overall enlarged heart. The viability of cardiomyocytes was significantly lower in the FASN-transgenic mice as TUNEL-staining for apoptotic cells confirmed. The metabolism of neonatal mouse cardiomyocytes showed an increased reliance on lipids for energy production, which is the predominant form of adult substrate use. Concerning lipid synthesis, higher concentrations of triglycerides and free fatty acids could be detected in 6-month old FASN-transgenic hearts. Levels of diacylglycerides and ceramides were also increased in FASN-transgenic hearts. These lipids are known to have potentially toxic effects on the cardiomyocyte (690) (694). Taken together the phenotyping of FASN-transgenic mice showed that a moderate increase in cardiac FASN protein is a sufficient and causative factor for the development of signs of heart failure.

**FASN-transgenic mice developed up-regulation of PPARG target genes**

Whole genome microarray gene expression profiling was performed to investigate mechanisms underlying the FASN-induced heart failure phenotype. The microarray gene expression study confirmed the shift towards a dysfunctional cardiac lipid metabolism, which was detected by the initial phenotyping. The GO analysis of the microarray data found an increased expression of genes involved in the pathologic lipid metabolism of heart failure. Classification of these genes by GO analysis identified a gene cluster of targets of the adipogenic transcription factor, PPARG (700). The PPARG gene cluster was significantly up-regulated more than 2-fold by transgenic FASN expression. These findings were considered relevant for the pathogenesis of heart failure because the PPARG target gene cluster was also found to be up-regulated in experimental models of heart failure, which mimic major cardiovascular risk factors, i.e. chronic pressure overload and atherosclerosis.

**Causal interplay between FASN and PPARG in the development of heart failure**

Tg-FASN mice showed upregulation of genes, which are targets of the transcription factor PPARG. In view of this finding, I wanted to investigate the direct relationship between PPARG and FASN in the development of heart failure. To upregulate PPARG target genes in vivo, I treated ApoE-deficient mice with the direct PPARG activator, rosiglitazone. Whole genome microarray gene expression profiling showed that rosiglitazone treatment resulted in the upregulation of the same genes which were also induced by transgenic FASN expression. Mice which were administered the PPARG activator rosiglitazone also developed signs of heart failure, and this heart failure phenotype was much more severe and appeared earlier when compared to untreated ApoE-deficient controls. In addition, the treatment with rosiglitazone induced a higher cardiac “lipid load”.

To investigate the impact of FASN activity on the PPARG activation-induced phenotype, I reduced activity of endogenous FASN in ApoE-deficient mice receiving rosiglitazone by expression of a FASN-specific small inhibitory RNA (si-RNA) by lentiviral transduction. Development of heart failure due to rosiglitazone was retarded and cardiac lipid load could be reduced by partial inactivation of FASN. These findings strongly indicated that FASN exerts a causal role in the development of PPARG activation-induced heart failure.
Isolated cardiomyocytes confirmed the causal interplay between FASN and PPARG

These in vivo findings with rosiglitazone-induced activation of Pparg in ApoE-deficient mice were complemented by studies with isolated rat neonatal cardiomyocytes. Expression of PPARG resulted in a more pronounced increase in free fatty acid production and reduced energy metabolism compared to that in non-transgenic cardiomyocytes. The effects induced by PPARG were enhanced by rosiglitazone. Cardiomyocytes expressing an overactive PPARG-S273A mutant, which cannot be phosphorylated and inactivated by ERK1/2, showed an increased accumulation of free fatty acids and a reduced ATP generation, even without rosiglitazone stimulation. Results derived from studies with isolated cardiomyocytes were confirmed by transgenic mice with PPARG-S273A expression, which showed a more severe cardiac phenotype compared to PPARG-transgenic mice. Signs of heart failure appeared earlier in mutant-PPARG-S273A mice and included cardiac hypertrophy and metabolic dysfunction.

To investigate the relationship between FASN and PPARG, the expression of FASN was down-regulated in PPARG- and mutant-PPARG-S273A-expressing cardiomyocytes by expression of a FASN-specific mi-RNA. Down-regulation of FASN reduced the free fatty acid concentration to moderate values, which were similar to measurements made in non-transgenic control rat cardiomyocytes. Concomitantly, there was an increased efficiency of the cardiomyocyte energy metabolism, resulting in an increased production of ATP. Taken together, my experiments showed that an increased PPARG activity and an increased FASN activity showed similar effects on energy metabolism and lipid production. Moreover, down-regulation of FASN in hearts and isolated cardiomyocytes with PPARG activation led to a decrease in pathological heart failure-related symptoms stressing that FASN mediates and promotes the activity of PPARG.

My findings could be relevant for the development of heart failure in patients because FASN is upregulated in human heart failure (674) (682). Regarding the underlying mechanism, my data showed that an increased PPARG activity and/or PPARG expression enhanced the activity of FASN. The up-regulation of FASN contributes to the deleterious effects of PPARG on cardiomyocyte energy metabolism and the development of heart failure because a decreased expression of FASN alleviated these harmful effects of PPARG overexpression. Because PPARG is also up-regulated in patients with heart failure and chronic pressure overload (724), my results indicate that a vicious circle between FASN activity and PPARG activity exists. Increased PPARG activity stimulates the expression of FASN while palmitate, which is the major lipid synthesized by FASN, activates PPARG. Both proteins stimulate each other in promoting heart failure.

GRK2 inhibition counteracted the FASN-induced cardiometabolic dysfunction

After having identified FASN and PPARG as two key players in the development of a dysfunctional cardiac metabolism and heart failure, I tried to find a treatment approach which reduced the activity of FASN and PPARG. Because the direct inhibition of FASN is lethal (682) (701) (702), I tried to target the increased FASN activity indirectly by modifying molecular signaling pathways influencing FASN and PPARG. The inhibition of GRK2 deemed to be a suitable choice for this purpose for several reasons. First, GRK2 levels are upregulated in heart failure patients (705). Second, GRK2 inhibits the ERK1/2 cascade, which in itself can promote the activity of PPARG (704) (711) whereas GRK2 inhibition enhances ERK1/2 activation in vitro and in vivo (512) (709). To study effects of GRK2 inhibition in vivo, we crossbred
transgenic mice with myocardium-specific overexpression of the peptide GRK(2) inhibitor, GRKInh (512), with transgenic mice overexpressing FASN.

Double-transgenic FASN+GRKInh mice showed a reduced cardiac lipid accumulation and improved energy metabolism in comparison with single-transgenic FASN counterparts. By inhibition of GRK2, the rate of beta-oxidation contributing to the total energy production in cardiomyocytes was normalized to levels measured in control cardiomyocytes. The FASN protein content of transgenic FASN hearts was also reduced by GRK2 inhibition, and accumulation of free fatty acids and triacylglycerides was significantly lower. Hypertrophy was reduced, heart function was improved, and less apoptotic cardiomyocytes could be detected in double transgenic FASN+GRKInh mice compared to single transgenic FASN-mice. The cardiometabolic function was protected by GRK2 inhibition and ATP generation could be improved.

I searched for mechanisms underlying these cardiometabolic improvements, which were induced by GRK2 inhibition in FASN-transgenic hearts. Several studies documented that PPARG activation is enhanced by ERK1/2 inhibition (704) (711). On the other hand, GRK2 inhibition enhances the activation of ERK1/2 (512) (709), which let us investigate this possible interaction by testing the effects of an MEK-ERK pathway inhibitor. The MEK inhibitor, PD0325901, stimulated PPARG activity in transgenic FASN-cardiomyocytes by inhibiting the Raf-ERK1/2 pathway. Vice versa, inhibition of GRK2 stimulated the activity of the Raf-ERK pathway and thereby inhibited PPARG. This effect resulted in a reduced expression of FASN and other PPARG target proteins. As a consequence, the inhibited PPARG activity and reduced production of palmitate by FASN led to a down-regulated expression of adipogenic genes to near-control level in double-transgenic FASN + GRKInh mice. Together these findings suggested that the enhanced activation of the Raf-ERK1/2 pathway induced by GRKInh was central to its cardioprotection activity.

**Dual inhibition of GRK2 and the Raf-Erk1/2 axis is not cardioprotective**

To further study the effects of the ERK1/2 pathway on PPARG and the cardiac energy metabolism, we examined the function of the Raf kinase inhibitor protein (RKIP) in transgenic mice. The RKIP is a dual-specific inhibitor, which inhibits GRK2 and the Raf-ERK1/2 pathway (689). The cardiomyocyte-specific overexpression of RKIP, which inhibits not only GRK2 but also the Raf-ERK1/2 pathway, had no beneficial effects and showed upregulation of several adipogenic genes involved in the development and promotion of heart failure, which included FASN and UCP1. These genes are known to be upregulated by ERK1/2 inhibition, which is caused by RKIP. Histological analysis showed a higher lipid content in transgenic RKIP hearts and functional cardiac assessment showed a reduced ejection fraction. Moreover, RKIP-transgenic isolated cardiomyocytes showed a dysfunctional metabolism typical of heart failure which was confirmed in vivo.

These experiments with RKIP-transgenic mice showed that the ERK1/2 axis is a central player, which can modify the activity of PPARG and its target genes. The loss of this regulatory mechanism is harmful for cardiac function. Furthermore, inhibition of GRK2 did not have positive effects on heart function when the ERK1/2 pathway was compromised.
GRKInh protected against the PPARG-induced cardiac dysfunction in vivo

To further investigate the effect of GRK2 inhibition on PPARG-induced symptoms of heart failure, I generated double-transgenic mice overexpressing PPARG and GRKInh. Double-transgenic PPARG+GRKInh mice developed less cardiac hypertrophy than their single-transgenic counterparts over-expressing only PPARG. The cardiomyocyte rate of beta-oxidation for energy generation was kept to near normal levels in the double-transgenic mice, while it was clearly elevated in PPARG-transgenic mice. These data are complementary to a previous study with PPARG-transgenic mice, which also showed that PPARG overexpression in the heart promoted symptoms of heart failure accompanied by cardioliptotoxicity (697).

The effect of GRK2 inhibition by the peptide GRKInh on the overactive PPARG-S273A mutant was also investigated. Transgenic expression of GRKInh showed weaker beneficial effects in transgenic mice overexpressing mutant PPARG-S273A compared to double-transgenic PPARG+GRKInh mice. Specifically, double transgenic mutant-PPARG-S273A + GRKInh mice showed only mild effects of GRK2 inhibition on cardiac metabolism and development of cardiac hypertrophy. These findings possibly indicate that the interplay between PPARG and FASN is disturbed by the PPARG-S273A mutation, which prevents the inactivation of PPARG by ERK1/2-dependent phosphorylation. Together my study showed that GRKInh can counteract the cardiometabolic dysfunction induced by wild type PPARG whereas the beneficial effects of GRK2 inhibition are strongly decreased in the PPARG-S237A mutant, which cannot be inactivated by ERK1/2.

In sum, my data suggest that beneficial effects of GRK2 inhibition rely on an intact Raf-ERK1/2 axis which protects against PPARG-induced cardiometabolic dysfunction.

GRK2 inhibition retarded the cardiometabolic dysfunction induced by chronic pressure overload

To investigate the relevance of the identified cardioprotective mechanism involving inhibition of PPARG target genes, I investigated the effects of GRK2 inhibition in a mouse model of cardiac pressure-overload induced by abdominal aortic constriction (AAC). While non-transgenic mice showed a high lipid accumulation within cardiomyocytes and developed severe cardiac dilative hypertrophy, transgenic GRKInh mice subjected to pressure overload by AAC developed less severe cardiac hypertrophy and had less lipid deposits in the cardiomyocytes, resulting in less cardiomyocyte apoptosis. The increased expression of adipogenic heart failure-related PPARG target genes that was seen in non-transgenic mice with cardiac pressure overload was significantly lower in transgenic mice with GRKInh and AAC.

These findings extend the relevance of the cardioprotective activity of GRK2 inhibition, which involves enhancement of the Raf-ERK1/2 axis, to a common cardiovascular risk factor, i.e. chronic pressure overload.

Identification of UCP1 as another player in cardiometabolic dysfunction

In search for additional targets regulated by the Raf-ERK1/2 axis, I focused on the uncoupling protein-1 (UCP1) because whole genome expression profiling revealed upregulation of the uncoupling protein-1 in cardiomyocytes of FASN-transgenic mice and in cardiomyocytes of mutant PPARG-S273A mice. In addition, in hearts subjected to pressure overload, Ucp1 was the most strongly upregulated adipogenic heart failure-related gene (own publication).
Moreover, several studies showed that UCP1 is up-regulated by inhibition of the Raf-ERK1/2 axis (704) (715). Those data were complemented by my gene expression data of RKIP-transgenic hearts, which also showed a strong up-regulation of UCP1 (own publication).Uncoupling proteins could be key players in the process of metabolic dysregulation in heart failure, because uncoupling proteins uncouple the process of proton flux from ATP generation in the mitochondria. Thereby they further reduce the efficiency of the already disturbed cardiac metabolism in heart failure in producing ATP which is a prerequisite for the heart’s efficient work.

In order to study the effects of UCP-1 up-regulation, I generated single-transgenic mice overexpressing UCP-1, which developed cardiac dysfunction with reduced ejection fraction, partially resulting from severe dilatation and loss of muscle mass. The cardiac ATP content in cardiomyocytes of UCP1-transgenic mice was significantly lower than in non-transgenic control mice, indicating increased uncoupling. Hypoxia-induced cardiac genes coding for the PPARG targets FASN and ACACA, were upregulated and the cardiac content of FASN protein was higher in UCP1-transgenic mice, probably due to an increased mitochondrial oxygen demand to ensure respiratory oxidation and ATP generation to compensate for increased uncoupling of the respiratory chain.

Inhibition of UCP1 by RNA interference in mice with pressure overload induced by abdominal aortic constriction, resulted in better cardiac function. In addition, this treatment resulted in improved energy metabolism with higher ATP production and less accumulation of free fatty acids in the cardiomyocytes overexpressing PPARG or overactive PPARG-S273A after transfection.

Results indicate that inhibition of GRK2 may be able to regulate the activity of PPARG and FASN. In addition, inhibition of UCP1 could also contribute to the improved cardiac function and metabolism, reducing or preventing heart failure caused by metabolic syndrome (FASN and PPARG models) or pressure overload (AAC). My data could also suggest that inhibition of UCP1 improved symptoms of ischemia.

Summary and Outlook

By studying the effects of FASN overexpression on several levels, I found that FASN activity is influenced by the activity of the peroxisome proliferator activator receptor gamma (PPARG) and the G-protein coupled receptor kinase 2 (GRK2).

My new findings identified a vicious circle of heart failure, which showed that GRK2 inhibition counteracts the activity of the peroxisome proliferator-activated receptor gamma (PPARG) by ERK1/2-dependent inactivation of this heart failure-promoting and adipogenic transcription factor. The vicious circle was not only active in FASN-transgenic hearts, but also contributed to signs of heart failure in an experimental model in which heart failure was induced by chronic pressure overload, a major risk factor of heart failure in patients. Therefore, my findings could also be relevant for the pathogenesis of chronic heart failure in patients.

Another major finding was the identification of a potential treatment approach, which interferes with this major heart failure-inducing pathomechanism. GRK2 is a druggable target, which can be targeted by small molecule compounds. By drug repurposing, the antidepressant paroxetine was identified as a lead structure for a small molecule GRK2 inhibitor. Based on this structure,
my ongoing efforts aim at identifying a selective GRK2 inhibitor with favorable pharmacokinetic properties, which could be tested in patients.
7. References


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8. Curriculum vitae

Curriculum Vitae

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born on 08.10.1979 in Hamburg, Germany, married, 1 child

University studies
10/2001-11/2009  Univ. study of human medicine, Johannes Gutenberg-University Mainz, Germany
10/2004  First federal medical exam (Physikum)
2004-2007  Training in ophthalmology, internal medicine, anesthesiology and psychiatry
2/2007-10/2007  1st experimental part of medical thesis „Microarray gene expression analysis of ApoE-deficient mice after treatment with the ACE inhibitor captopril (supervisor: Prof. Dr. Ulrich Förstermann, Institute of Pharmacology, Univ. Mainz; external supervisor: Prof. Dr. Ursula Quitterer, ETH Zurich)
2/2008-1/2009  Clinical internship, Horst-Schmidt-Kliniken Wiesbaden (surgery, internal medicine, dermatology)
12.11.2009  Second federal medical exam and German approbation as physician
20.04.2011  Swiss medical diploma approval by MEBEKO
07/2014  FMH membership

Medical thesis (MD)
03/2012  MD exam; graduation Dr. med. (‘‘magna cum laude‘‘ - very good)

Professional experience
01/2010 - 08/2012  Ass. I, Mol. Pharmacology Unit, ETH Zurich
09/2012 - 11/2012  Physician, Clinic for traumatology, Univ. Hospital, Zurich
12/2012 - 04/2013  Ass. II, Mol. Pharmacology Unit, ETH Zurich
05/2013 - 05/2014  Physician, University Center for Medicine of Aging at the Felix Platter-Hospital, Basel
since 01/2013  Ph.D. thesis in Molecular Pharmacology, ETH Zurich
since 06/2014  Ass. II, Molecular Pharmacology, ETH Zurich
### Additional training

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<tr>
<td>09/2006</td>
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<td>03/2007</td>
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9. List of Publications

List of publications (past 5 years)
Dr. med. Joshua Abd Alla

I. Patent application

1. **Abd Alla J**, Graemer M, Wolf S, Quitterer U. Cardioprotective compounds and their use (ETH 2016-138), Application number EP17150829.4, date of receipt 10 January 2017. (ETH Technology evaluation index **83 out of 100**).

II. Peer-reviewed publications


   “Paper of the Week” (top 2 % of more than 6600 articles) published in the *J Biol Chem* per year.


III. Other publications (not peer-reviewed)


3. **Abd Alla J**, Graemer M, Quitterer U. Transgenic mice with myocardium-specific over-expression of fatty acid synthase (FASN) develop signs of cardiac hypertrophy and failure. *Naunyn-Schmiedeberg’s Arch. Pharmacol.* 387 (Suppl. 1), S88 (2014).


### IV. Whole genome microarray gene expression studies (NCBI GEO database)


   **Selected by GEO as GEO Profile (GDS4544)**


### V. Oral and poster presentations at scientific meetings


6. **Abd Alla J,** Graemer M, Quitterer U. Transgenic mice with myocardium-specific over-expression of fatty acid synthase (FASN) develop signs of cardiac hypertrophy and failure. Poster. 80th Annual Meeting of the German Society of Experimental and Clinical Pharmacology and Toxicology, Hannover, Germany, April 1 - 3 (2014).


10. Abbreviations

2-DG 2-Deoxy-D-glucose
7TMR Seven transmembrane receptor
AAC Aortic abdominal constriction
ACAT1 Acetyl CoA-transferase
ACC American College of Cardiology
ACCF American College of Cardiology Foundation
ACE Angiotensin-converting enzyme
Acetyl-CoA Acetyl coenzyme A
ACS Acute coronary syndrome
acyl-CoA Acyl coenzyme A
AD Alzheimer's disease
ADRBK1 Beta adrenergic receptor kinase 1, synonym GRK2
AHA American Heart Association
AMP/ATP ratio Adenosine monophosphate/adenosine triphosphate ratio
AMPK Adenosine monophosphate-activated protein kinase
ANP Atrial natriuretic peptide
AP-1 Activator protein 1
ApoE Apolipoprotein E
ARB Angiotensin receptor blocker (angiotensin II receptor antagonist)
ARIC study Atherosclerosis risk in communities study
ARNI Angiotensin receptor-neprilysin inhibitor
ARRB1 Arrestin beta 1
AT1R Angiotensin II receptor type 1
AT2R Angiotensin II receptor type 2
ATP Adenosine triphosphate
BARK Beta adrenergic receptor kinase, synonym GRK2
BARK C-terminus carboxy-terminus of beta adrenergic receptor kinase
BMI Body mass index
BNP Brain natriuretic peptide / B-type natriuretic peptide
CAC Coronary artery calcium score
CAD Coronary artery disease
CCR Chemokine receptor 9
CCT Coronary computed tomography
CD 36 Cluster of differentiation 36
cGMP Cyclic guanosine monophosphate
CHD Coronary Heart Disease
CHF Congestive heart failure
CMR Cardiovascular magnetic resonance imaging
COX-2 Cyclooxygenase 2
CPT-1 Carnitine palmitoyltransferase 1
CREB cAMP response element-binding protein
cRNA complelementary RNA
CTA Computed tomography angiography
<table>
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<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
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<tr>
<td>IU</td>
<td>International unit</td>
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<td>JAM-C</td>
<td>Junctional adhesion molecule C</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>KCCQ</td>
<td>Kansas City cardiomyopathy questionnaire</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
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<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
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<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
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<td>LVD</td>
<td>Left ventricular dysfunction</td>
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<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
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<td>MAC</td>
<td>Membrane attack complex</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCP-1</td>
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<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
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<td>MEK inhibitor</td>
<td>Mitogen-activated protein kinase kinase inhibitor</td>
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<td>MHC</td>
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<td>MHC promoter</td>
<td>Alpha-mysin heavy chain promoter</td>
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<td>MI</td>
<td>Myocardial infarction</td>
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<td>miRNA</td>
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<td>Monocyte to macrophage differentiation associated (protein)</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MPI</td>
<td>Myocardial perfusion imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NAD</td>
<td>nicotinic adenine dinucleotide</td>
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<tr>
<td>nCEH</td>
<td>neutral cholesterol ester hydrolase 1</td>
</tr>
<tr>
<td>NF-kappa B</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NHANES</td>
<td>National health and nutrition examination survey</td>
</tr>
<tr>
<td>NICE guidelines</td>
<td>National institute for health and care excellence guidelines (UK)</td>
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<tr>
<td>NIH</td>
<td>National institutes of health (US)</td>
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<tr>
<td>NP</td>
<td>Natriuretic peptide</td>
</tr>
<tr>
<td>NPR-A</td>
<td>Natriuretic peptide receptor A</td>
</tr>
<tr>
<td>NRF-1</td>
<td>Nuclear respiratory factor 1</td>
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<tr>
<td>NSDUH</td>
<td>National survey on drug use and health</td>
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<tr>
<td>NYHA</td>
<td>New York heart association</td>
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<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PDC</td>
<td>Pyruvate decarboxylase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase 4</td>
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<tr>
<td>PECAM</td>
<td>Platelet endothelial adhesion molecule</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
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<tr>
<td>PGC-1a</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
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<tr>
<td>PH domain</td>
<td>Pleckstrin homology domain (GRK2)</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
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<tr>
<td>PKB/Akt</td>
<td>Protein kinase B</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>plasma TAS</td>
<td>Plasma total antioxidant status</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<tr>
<td>PPARG</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
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<tr>
<td>PTP1B</td>
<td>Protein-tyrosine-phosphatase 1B</td>
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<tr>
<td>RAS</td>
<td>Renin angiotensin system</td>
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<td>RCT</td>
<td>Randomized controlled trial</td>
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<td>RGJ</td>
<td>Red grape juice</td>
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<tr>
<td>RH domain</td>
<td>Regulator of G-protein signaling homology domain (GRK2)</td>
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<tr>
<td>RKIP</td>
<td>Raf kinase inhibitor protein</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<td>RXR</td>
<td>Retinoid X receptor</td>
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<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>Thiol group</td>
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<tr>
<td>SHRSP rat</td>
<td>Spontaneously hypertensive stroke prone rat</td>
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<tr>
<td>SIHD</td>
<td>Stable ischemic heart disease</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>Siruin 1</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>Sprr3</td>
<td>small proline-rich protein 3</td>
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<tr>
<td>STEMI</td>
<td>ST-segment elevation myocardial infarction</td>
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<tr>
<td>TAG</td>
<td>Triacylglyceride</td>
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<tr>
<td>TC</td>
<td>Total cholesterol</td>
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<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
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<tr>
<td>TCC</td>
<td>Terminal complement complex</td>
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<tr>
<td>Tg</td>
<td>Transgenic (adjective)</td>
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<td>TGF-beta</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF-alpha</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-related ligand</td>
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<td>Terminal deoxnucleotidyl transferase dUTP nick end labeling assay</td>
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<tr>
<td>UA</td>
<td>Unstable angina</td>
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<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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