Metoprolol increases TIMP-2 expression in mice bearing acute complex atherosclerotic plaque

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1. Introduction

1.1 Atherosclerosis

Atherosclerosis is often assumed to be a new age disease. However, there is evidence that ancient human beings were already suffering from atherosclerosis. The disease has been found in mummies from different regional populations spanning over 4000 years of history.\(^1\) Atherosclerosis is the underlying cause for several cardiovascular diseases such as myocardial infarction (MI) or stroke that both cause high mortality rates in industrialized countries and worldwide. It has been reported that in the United States more than 22% of deaths result from coronary heart disease or stroke in 2006. According to the 2014 report of the American Heart Association (AHA) in the United States an average of 1 death every 40 seconds occurs due to cardiovascular events.\(^2\) Cardiovascular diseases altogether accounted for 31.3% deaths in the United States in the 2015 report of the AHA.\(^3\) Also, atherosclerosis causes a large economic and public health impact.\(^4\) The direct and indirect cost for treatment of coronary heart disease and stroke are estimated to consume 250.8 billion dollar in the United States, which places it at the top of the list of all diagnostic groups.\(^5\)

Atherosclerosis is a life long disease that can remain silent, without obvious clinical symptoms for decades. Fatty streaks that sometime can already be found during childhood are the initial step of developing atherosclerotic plaques. During adulthood those then matured lesions are held accountable for cardiovascular complications by precipitating cardiovascular events such as MI or stroke.\(^6\) The common view of atherosclerosis is a slow and chronic lipid accumulation in the vessel wall that among other causes can be due to high
cholesterol levels and pathological lipoprotein profiles. The initial step of atherosclerotic disease development is thought to be an injury to the endothelium that can be caused by for example dyslipidemia. Increased plasma levels of lipoproteins, especially low-density lipoprotein (LDL), are sufficient for the initiation of atherosclerosis in humans. However, numerous other risk factors are also known to influence the progression of atherosclerosis by impairing endothelial function. These factors can generally be divided into i) environmental and ii) hereditary factors. The environmental factors include smoking, physical inactivity, and malnutrition, while gender, hypercholesterolemia, high blood pressure, metabolic syndrome, diabetes mellitus and chronic kidney disease have hereditary components.  

1.2 Atherosclerosis and inflammation

Nowadays, atherosclerosis is considered to be a complex systemic, chronic and progressive inflammatory disease of the arterial wall leading to atherosclerotic plaque development. It promotes thickening of the vessel wall and the generation of an atheroma that can protrude into the arterial lumen, thus causing blood flow limitations with the inherent complications. Ross’s hypothesis of the injured endothelium for the first time described that atherosclerosis initiates with an injury of the endothelium which is followed by the adhesion and aggregation of platelets and leukocytes. However, the current understanding of initiation of atherosclerosis has evolved. At present, the initial step in the process of atherosclerotic lesion development is seen as an endothelial dysfunction rather than a morphologically relevant endothelial injury.
In spite of the common view of atherosclerosis as a chronic disease of lipid accumulation in the vessel wall due to high cholesterol levels and lipid profiles, atherosclerosis is a dynamic disease with several series of cellular and molecular changes and signaling pathways involved, all funneling into the diseased vessel wall. Healthy endothelium is not a simple lining of cells covering the internal surface of blood vessels, but plays an important role to inhibit leukocyte adhesion and migration and limit platelet adhesion. Hypercholesterolemia, hypertension, tobacco smoke, modified LDL, certain microorganisms and other risk factors for atherosclerosis cause endothelial activation in the arterial wall. This process is characterized by increased expression of a variety of surface adhesion molecules: E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) that bind receptors present on monocytes and T-cells. Once adherent to the endothelium, leukocytes migrate into the intima. Monocyte chemoattractant protein-1 (MCP-1) appears to be at least in part responsible for the recruitment of monocytes. A family of T-cell chemoattractants helps lymphocytes penetrate into the intima. Monocytes and T cells accumulate at the site of dysfunctional endothelium and penetrate the vessel wall. Inside the intima, monocytes mature into macrophages and start to express scavenger receptors including: scavenger receptor A1 (SR-A1), SR-A2, SR-B1, and CD36 to recruit modified lipoprotein particles like oxidized LDL (oxLDL). The uptake of oxLDL by macrophages under the influence of macrophage-colony stimulating factor (M-CSF) will lead to foam cell formation within the arterial wall. This in turn could eventually further increase inflammation by acting as a chemoattractant that increases
recruitment of additional macrophages into the vessel wall. Researchers defined this characteristic lesion as fatty-streak, which consists of macrophage foam cells and T-cells located under a layer of endothelial cells.¹³

Macrophages, which are present in the fatty-streak lesion, bind to and internalize oxLDL initiating differentiation into the M1-like pro-inflammatory phenotype (also known as classically activated macrophages) by secreting cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β). Other macrophages, under the influence of cytokines that are released to limit the chronic inflammation, attain the M2-like phenotype (alternatively activated macrophages). These express additional factors believed to benefit anti-inflammatory processes potentially involved on lesion regression.¹⁸

Macrophages may also leave the plaque either by reverse transmigration through the endothelium or by migrating from the media to the surrounding adventitia and then lymphatics.¹⁹

Antigens such as oxLDL or Heat Shock Proteins activate T-cells in the plaque. Some T-cell subsets differentiate into proinflammatory (Th1) cells, while others appear to attenuate inflammation (Th2 cells).²⁰ Cytokines secreted inside the plaque promote a Th1 response rather than a Th2 response. Activated T-cells secrete TNF-β, γ -interferon(IFN-γ ) and growth factors that can promote vascular smooth muscle cell (VSMCs) migration and proliferation.⁸,¹³ Figure 1 presents the recruitment and differentiation of inflammatory cells in atherosclerosis. The processes depicted result in an amplified inflammatory response, which can induce alterations of VSMCs towards a more migratory and proliferative phenotype. While the nascent steps of atherogenesis involve mostly
endothelial dysfunction, macrophage recruitment and activation, the evolution of
the atheroma toward a mature lesion also is characterized by participation of
VSMC. They migrate from the tunica media to the intima in response to different
chemoattractants including platelet-derived growth factor (PDGF) that is
secreted by activated macrophages. Meanwhile, VSMC secrete factors, which can
again recruit additional monocytes.

Figure 1: Inflammatory cells in atherosclerosis. This figure depicts steps in the
recruitment of mononuclear phagocytes and T-cells during the early stages of
atherosclerotic plaque formation and some of the functions of these cells in the mature
atheroma.

Modified from: Libby P. Nature 2002;420:868-874
If the inflammatory response and plaque growth are sustained, the vessel wall begins to remodel by gradual dilation in order to maintain the lumen size constant.\textsuperscript{6} The growing plaque will not be able to support viability of cells. Thus, apoptosis and necrosis of macrophages and SMCs are found in the core region of the lesion and combined with proteolytic and hydrolytic enzyme released from inflammatory cells, core necrosis can ensue.\textsuperscript{21,22}

\subsection*{1.3 Plaque categories}
Cardiologists traditionally consider coronary artery disease as severe when blood flow is limited by plaque and the lesion occupies more than 50\% of the arterial lumen. However, a number of angiographic and autopsy studies has identified that stenosis of less than 50\% accounted for almost 70\% of MI. These are due to rupture of plaques smaller than 50\% of lumen size (Type 1 MI), and only less than 20\% are due to severe stenosis and supply-demand mismatch (Type 2 MI).\textsuperscript{23,24} Thus, the quality of the plaque rather than its size determines the clinical consequences of atherosclerosis.\textsuperscript{25} Meanwhile, it has been recognized that the stability of atherosclerotic plaques is equally important.\textsuperscript{26}

In summary: While most of the human plaques remain asymptomatic, some become symptomatic as lined out above. Symptoms can be due to two characteristics of the plaque: Obstruction of blood flow or plaque rupture. Plaque rupture can precipitate different sequelae. Some become thrombosis-prone and lead to atherothrombotic events, such as acute myocardial infarction (AMI),\textsuperscript{27} stroke,\textsuperscript{10} and lower limb ischemia.\textsuperscript{28} To describe plaques prone to precipitate these complications they are separated into two broad categories: stable and unstable, the latter also called vulnerable plaques.
The pathobiology of atherosclerotic plaques is complicated. Somewhat artificially, plaques can be divided into the following groups: An atherosclerotic plaque with a thick, intact fibrotic cap containing smooth muscle cells and with high collagen content is considered to be a stable plaque.\textsuperscript{29,30} This plaque has few macrophages, low lipid content, and proinflammatory cytokine expression. The vulnerable plaques present histological features like a large lipid core, a thin fibrous cap, low collagen and smooth muscle cell content, increased expression of proinflammatory cytokines and an imbalance between extracellular matrix synthesis and degradation.\textsuperscript{6,26} Fibrous cap thickness, lipid core size, and plaque cell composition are the main factors that determine the stability of the plaques. A vulnerable plaque with thin atherosclerotic cap can rupture either spontaneously or due to triggers such as increased heart rate and blood pressure that will impose mechanical strain. Ruptures primarily occur in the shoulder regions of plaques. Upon rupture prothrombotic material of the plaque interior is presented to the blood stream. Blood in patients bearing large loads of plaques is in a hypercoagulable state and will readily form platelet rich thrombi that can occlude the vessel or embolize into the periphery to cause vascular occlusion there.\textsuperscript{31}

Thinning of the cap can happen due to two reasons: one is the presence of macrophages secreting matrix metalloproteinases (MMPs), which can degrade the extracellular matrix and render the cap thin and prone to mechanically induced disruption and the other is the loss of vascular smooth muscle cell mass in the cap of the plaque. The caps are usually the thinnest in the shoulder regions, where often macrophages accumulate and become activated. Consequently most
of the ruptures of unstable plaques happen at this site. Rupture of the fibrous cap induces thrombus formation on the plaque surface and can result in MI or stroke. About 75% of the thrombi that are responsible for the following acute coronary syndrome build up after a rupture of a small, yet vulnerable plaque. These thrombi obstruct the vessel, prevent blood flow and thus cause ischemia of the tissues nourished by the respective artery.\textsuperscript{32,33}

In order to study vulnerable plaques in depth, a histopathologic definition of unstable plaques has been described according to the ground work by Herbert Stary and his colleagues.\textsuperscript{34-36} Using these classical criteria, plaques are divided into six types starting from type I, with complex plaques grouped in one single category VI.(Figure 2) Lesion types from I to III are considered as early lesions, which can potentially reverse. While lesion types IV to VI are named atheroma, fibroatheroma and complicated lesion. Type I lesions are defined as isolated macrophages containing lipid droplets, which are also called foam cells. Most of type I lesion are not visible without microscopy techniques. This type has also been named „initial lesion“. Type II lesions contain clusters of macrophage foam cells bedded in adjacent layers of cholesterol esters. This lesion type is also called fatty streak. But not all type II lesions present visibly as a fatty streak. Type III lesions present with the feature of visible extracellular lipid droplets and particles under the microscope that form pools. This lesion’s layers induce intimal thickening. Therefore, type III lesions are also recognized as intermediate lesions. The typical characteristic is the presence of a lipid core in lesion type IV, which is also known as atheroma and considered as the first advanced lesion. Type V lesion is described as lipid core connected with new fibrous layers. Calcified and fibrotic material is the main part of the lesion. So this lesion is also
referred to as fibroatheroma. Type VI lesion, as stated above, is characterized by surface disruption, or hemorrhage and/or thrombosis, and is known as complicated lesion.

<table>
<thead>
<tr>
<th>Nomenclature and main histology</th>
<th>Sequences in progression</th>
<th>Main growth mechanism</th>
<th>Earliest onset</th>
<th>Clinical correlation</th>
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<tbody>
<tr>
<td>Type I (initial) lesion</td>
<td></td>
<td>growth mainly by lipid accumulation</td>
<td>from first decade</td>
<td>clinically silent</td>
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<td>isolated macrophage foam cells</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Type II (fatty streak) lesion</td>
<td></td>
<td>growth mainly by lipid accumulation</td>
<td>from third decade</td>
<td></td>
</tr>
<tr>
<td>mainly intracellular lipid accumulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type III (intermediate) lesion</td>
<td></td>
<td>accelerated smooth muscle and collagen increase</td>
<td>from fourth decade</td>
<td>clinically silent or overt</td>
</tr>
<tr>
<td>Type II changes &amp; small extracellular lipid pools</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IV (atheroma) lesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II changes &amp; core of extracellular lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type V (fibroatheroma) lesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipid core &amp; fibrotic layer, or multiple lipid cores &amp; fibrotic layers, or mainly calcific, or mainly fibrotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type VI (complicated) lesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>surface defect, hematoma-hemorrhage, thrombus</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 2: Diagrammatic sketch of the histopathologic category of atherosclerotic lesion provided by AHA.

1.4 Atherosclerosis during the perioperative phase

It is estimated that 40 million surgical procedures are performed every year in Europe with a post-operative MI rate of 1% (400,000), and a cardiovascular mortality rate of 0.3% (133,000). Patients with preexisting cardio-vascular disease undergoing major surgical procedures are at high risk for plaque rupture in the perioperative period. Plaque rupture in this situation can cause fatal perioperative myocardial infarction (PMI) or stroke. According to the pathological, clinical and prognostic differences and different treatment
strategies, MI is subdivided into 5 types.\textsuperscript{42} (Table 1) PMI is the predominant cause of morbidity and mortality in patients undergoing non-cardiac surgery.\textsuperscript{24} The pathophysiology of PMI is related to perioperative stress, inducing imbalance of oxygen supply-demand in the presence of a coronary artery stenosis or a sudden coronary plaque rupture with thrombosis and vessel occlusion, which is different from MI patients without experiencing surgery. In addition, perioperative stress increases myocardial oxygen demand, which in the presence of an increased heart rate with shorter perfusion times and increased coronary blood flow reduces myocardial oxygen supply.\textsuperscript{24} Critical supply reduction can lead to supply-demand mismatch of coronary blood flow and thereby induce tissue ischemia and MI.\textsuperscript{43,44} Perioperative stress precipitates myocardial ischemia that, different from the non-surgical setting, in equal proportions originate either from rupture of an unstable atherosclerotic plaque or from coronary blood flow obstruction in the face of increased demand.\textsuperscript{33,43,45,46}

Table 1. Universal classification of myocardial infarction

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>spontaneous myocardial infarction</td>
</tr>
<tr>
<td>Type 2</td>
<td>myocardial infarction secondary to an ischemic imbalance</td>
</tr>
<tr>
<td>Type 3</td>
<td>myocardial infarction resulting in death when biomarker values are unavailable</td>
</tr>
<tr>
<td>Type 4</td>
<td>myocardial infarction related to percutaneous coronary intervention or stent thrombosis</td>
</tr>
<tr>
<td>Type 5</td>
<td>myocardial infarction related to coronary artery bypass grafting</td>
</tr>
</tbody>
</table>

Therapeutic interventions are limited in the perioperative phase because of the unpredictable progression of a non-significant coronary lesion to plaque rupture, thrombus formation, and subsequent coronary artery occlusion. Detection of these lesions prior to surgery is still not easily possible.\textsuperscript{37} PMI induces a much higher lethality than MI without preceding surgery. Reduction of perioperative myocardial ischemia reduces the risk for MI and improves outcome.\textsuperscript{43} Systemic
therapy with medical treatment aiming at plaque stabilization therefore seems promising for perioperative as well as long-term risk reduction. However, current prophylactic strategies are limited and can prevent only a minority of perioperative cardio-vascular events.

1.5 $\beta$-adrenergic receptor antagonist in perioperative medical management

$\beta$-adrenoceptors are a group of the seven-transmembrane helix receptors. They belong to the family of G-protein-coupled receptors. Nowadays three types of $\beta$-adrenoceptors are known, which are designated $\beta_1$, $\beta_2$, and $\beta_3$ receptors, classically recognized in cardiac, airway smooth muscle and adipose tissue respectively. The recognition of these subtype receptors give rise to the evolution of selective agonists and antagonists. Overall, catecholamines predominantly bind to $\beta_1$-adrenoceptors. Epinephrine and norepinephrine release and engagement of the receptor result in positive inotropic and chronotropic effects to increase cardiac output. $\beta_2$-adrenoceptor activation mainly induces smooth muscle relaxation. $\beta$-agonists bind to $\beta$-adrenoceptors and imitate the actions of sympathetic system. $\beta_1$-adrenoceptors have similar affinities for epinephrine and norepinephrine, while $\beta_2$-adrenoceptors have higher affinity for norepinephrine than for epinephrine. $\beta_1$-selective blockers, such as atenolol, bisoprolol, metoprolol etc, are considered cardioselective. Their action results in a heart rate decrease and a reduction of myocardial contractility. The older generation of non-$\beta_1$-selective blockers have members like propranolol. They have additional effects through the blockade of $\beta_2$-adrenoceptors.
β-adrenergic receptor antagonist therapy plays a major role in the treatment of cardiovascular diseases. β-adrenoceptor blockers (BRB) were originally brought to clinical use in the 1960s and for many years they have been used for a variety of approved indications including angina pectoris, hypertensive control, cardiac arrhythmia management, post-coronary revascularization mortality reduction, and post MI risk reduction. BRB block the action of endogenous catecholamines epinephrine and norepinephrine in particular. Perioperatively, the rise of endogenous catecholamine levels leads to increased heart rate and myocardial contractility resulting in increased oxygen demand. β-adrenoceptor blocking agents restore the oxygen supply-demand mismatch by reducing myocardial oxygen consumption.

In the context of myocardial infarction, which represents a state of reduced oxygen supply to the affected portion of the heart, these effects may be beneficial as BRB result in reduced myocardial workload and oxygen demand. For patients with AMI, BRB therapy reduces infarct size and early mortality when started early and lowers the risk of death when continued long term. BRB have been shown to reduce recurrent MI, sudden cardiac death and total mortality in patients with AMI in several clinical trials. Perioperative BRB have become standard therapy in the prevention of MI in patients undergoing non-cardiac surgery. Wide acceptance based on a series of very promising randomized controlled trials was however followed by a number of critical papers. Meanwhile, the recommendations to broadly administer BRB for perioperative cardioprotection have been revised. However, their specific effect on reducing cardiovascular events is undisputed and was also seen in the
trials demonstrating that adverse effects consume the benefit if the substances are inadequately dosed or indicated. Most of the authors suspect the hemodynamic effects of the drugs to be responsible for the long-term beneficial effects of BRB. If these substances also exert a direct anti-atherosclerotic effect has been examined in several clinical studies that observed anti-atherosclerotic effect of long-term treatment with metoprolol in the atherosclerotic process. A recent pooled analysis of four intravascular ultrasonography trials has shown that BRB slow the progression of atherosclerosis. Many animal experiments have also provided evidence that BRB can slow the development of atherosclerosis. If BRB can prevent plaque destabilization in acutely proinflamamtory and stressful situations as the perioperative phase has never been examined.

1.6 Murine models of atherosclerosis

A variety of small and large animal models have been used to study atherosclerosis and explore novel therapeutic strategies. Currently the mouse is the most frequently used species for atherosclerosis studies. Advantages are the rapid generation time, disease development kinetics and relatively low cost of maintenance. But first and foremost like in no other species the ease of genetic manipulation allows for transgenesis, gene knockout and knockin, as well as for temporal and tissue specific conditional knockout or expression of genes. Apolipoprotein E-deficient (ApoE−/−) and LDL receptor-deficient (LDLr−/−) knockout mice are the two most frequently used mouse models of atherosclerosis. They differ in their dietary needs for the development of atherosclerosis. The most important characteristic of ApoE−/− mice is that they can develop
spontaneous atherosclerosis, which can be also substantially accelerated by a high fat diet. The developing plaques somewhat resemble human plaques but do not rupture except from the innominate artery branching point or surgical models.\textsuperscript{70} LDLr\textsuperscript{-/-} mice develop atherosclerosis somewhat slower on normal diet. Although studies in murine models have significantly contributed to our understanding of the mechanisms of atherogenesis, a shortcoming in these models is that plaque rupture, with superimposed thrombosis commonly observed in humans, is rarely observed in these two mouse models\textsuperscript{69,71} unless surgery is applied on the mice.\textsuperscript{72-74} Unlike humans, mice develop atherosclerosis in the coronary arteries only at very late stages, but readily develop atherosclerosis in the aortic root.\textsuperscript{69} When designing an atherosclerosis research project the site of examination of the atherogenic process is of utmost importance. There are some studies showing that very particular plaque-prone sites provide better information with respect to certain plaque features than others.\textsuperscript{75} The key problem in mouse models for atherosclerosis is the lack of plaque rupture in these rodents. Even with very advanced plaques obstruction of blood flow due to plaque rupture and thrombus apposition has never been reported. The debate about the usefulness of mouse models to test plaque stabilizing strategies for human disease is controversial.\textsuperscript{69,70} Jason Johnson for the first time describes plaques bearing signs of plaque vulnerability and rupture in ApoE\textsuperscript{-/-} mice.\textsuperscript{76} These features are exclusively present in the innominate artery plaque at its branching point from the aorta and occurred at a very particular point in time during high fat feeding. Recently, we first developed a novel model of postoperative plaque destabilization mimicking perioperative stress by the combination of a laparotomy with acute loss of 20% blood volume in general
17 anesthesia. This new double hit model demonstrates that a short period of perioperative stress can promote plaque growth and induces signs of plaque instability.

1.7 Matrix metalloproteinases in atherosclerosis

MMPs are a family of zinc containing extracellular matrix degrading proteinases, produced by a variety of cells including endothelial cells, macrophages, and smooth muscle cells. MMPs are capable to break down extracellular matrix (ECM). Their activity is inhibited by a family of antagonists called tissue inhibitors of MMP (TIMPs). MMPs and TIMPs play a major role not only in ECM degradation but also in mediating cell migration, proliferation and tissue remodeling. A substantial body of evidence supports the notion that an imbalance between the activity of MMPs and TIMPs contributes to the pathogenesis of cardiovascular diseases such as atherosclerosis and vascular remodeling but also worsens the progression of heart failure due to adverse ventricular remodeling.

MMPs are believed to contribute to the development and progression of atherosclerosis. Several MMP deficient and TIMP transgenic mice show decreased atherosclerosis progression or more stable plaque phenotypes. High levels of MMP-9 activity promotes instability of plaque, especially in advanced plaques provoked by diet induced excessive hypercholesterolemia. Kuzuya M et al demonstrate that MMP-2 deficiency reduces atherosclerotic plaque lesions in ApoE-/- mice. It has been reported that overexpression of TIMP-1 reduces atherosclerotic lesions in ApoE-/- mice. However, atherosclerotic lesion size and stability are unaffected in the aortas of mice deficient for TIMP-1. Johnson et al
suggest that TIMP-2 is an effective inhibitor of atherosclerotic plaque growth and promotes a stable plaque phenotype in the fat-fed ApoE/− mice brachiocephalic artery, they suggest that overexpression of TIMP-2 but not TIMP-1 inhibits atherosclerotic plaque development and destabilization.85

It's well known that long time exposure to catecholamines may contribute to vascular and cardiac remodeling in experimental animal models and in man. In addition, researchers demonstrate that this process may be modulated by β-adrenoceptor signaling resulting in expressional effects on the MMP system.86 Isoprenaline is known as a non-selective β-adrenoceptor stimulator, which increases MMP-1, MMP-2 and MMP-9 expression in rats.87-89 However, β1-adrenoceptor but not β2-adrenoceptor antagonists abolish this effect.90 In 2000, Senzaki and his colleagues first reported that a β1-adrenoceptor antagonist reduced MMP-9 expression in pigs with heart failure. Since then, there is ample evidence suggesting that the expression of MMPs increases due to the release of the catecholamines epinephrine or norepinephrine, and this effect can be attenuated by β-adrenoceptor antagonists.91

1.8 Purpose and hypothesis

The overall theme of this study is the notion that attenuation of hemodynamic stress (heart rate; blood pressure) using β-adrenoceptor antagonists can reduce the incidence of MI in the perioperative phase. It is thought that BRB reduce myocardial oxygen consumption, block the sympathetic system, and decrease heart rate and blood pressure.53,92,93 The effect of hemodynamic control by BRB on acute plaque composition and stability has never been examined. Therefore this study was designed to investigate the direct, short-term effects of
hemodynamic control by BRB on plaques size and composition using a mouse model of plaques in mice that are exposed to a surgical procedure combined with blood loss. We also tested if BRB have effects on plaque size and complexity independent of their hemodynamic effects.

We thus tested the following hypothesis: Hemodynamic control by β-adrenoceptor antagonist utilized in the perioperative phase attenuate acute changes of atherosclerotic plaque size and reduce signs of instability in a mouse model. We further hypothesized that hemodynamic independent effects are mediated through the MMPs system.
2. Materials and Methods

2.1 Chemicals, antibodies, consumables and equipment

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goat anti-mouse TIMP2 R&D System, Germany

goat anti-rat IgG Jackson ImmunoResearch, West Grove, USA

goat anti-mouse IgG Jackson ImmunoResearch, West Grove, USA

Fluorescent Mounting Medium DAKO, Germany

Tissue Freezing Medium Leica, Germany

TRItidy® reagent Applichem, Darmstadt, Germany

Primers Eurogentec Cologne, Germany

SensiFAST TM SYBR Bioline GmbH, Germany

Novex 10% gelatine zymogram gels Invitrogen, USA

Novex Running buffer Invitrogen, USA

NovexRenaturing buffer Invitrogen, USA

Cryomold Tissue-Tek Science Services Germany

Prolenemonofilblau 5/0 Ethicon, Germany

Superfrost microscope slides Menzel-Gläser,Braunschweig, Germany

Disposable needles B. Braun AG, Melsungen, Germany

Syringes 1 ml, 5 ml BD, Heidelberg, Germany

Gloves B. Braun AG, Melsungen, Germany

15ml tubes Sarstedt, MedLager, Germany

50ml tubes Sarstedt, MedLager, Germany

Single-channel pipettes

- Research 2.0-20μl Eppendorf AG, Hamburg, Germany

- Research 10-100μl Eppendorf AG, Hamburg, Germany

- Research 100-1000μl Eppendorf AG, Hamburg, Germany
Pipette tips
- Blue, 100-1000μl Eppendorf AG, Hamburg, Germany
- Yellow, 1-100μl Eppendorf AG, Hamburg, Germany
- White, 0.1-10μl Eppendorf AG, Hamburg, Germany

Pipette boy Pipetus® Hirschmann, Eberstadt, Germany

Centrifuge Eppendorf AG, Hamburg, Germany

tail cuff device AD Instruments, Marburg, Germany

Microtome Osaka, Japan

Infinite M200 multiwell reader TECAN, Männedorf, Swiss

Photometer Eppendorf AG, Hamburg, Germany

Biometra Cycler Göttingen, Germany

RT-PCR cycler Rotogene 3000 Corbett Life Science, Hilden, Germany

Olympus IX81 microscope Olympus, Hamburg, Germany

Cell-F version 3.1 Olympus, Hamburg, Germany

ImageJ 1.48v analysis software NIH, USA

Prism 5 GraphPad Prism, USA

2.2 Double hit model, drug treatment and tissue harvesting

Perioperative stress is caused by a combination of the surgical trauma, pain and fluid shifts due to hemorrhage and barrier disturbances. 7 weeks of diet induce small fatty streak lesions and allow investigation of net increases of plaque size. We hypothesized that 10 weeks of diet would induce advanced lesions bearing signs of vulnerability and allow to examine the effect of interventions on established lesions, i.e. regression of plaques.
Experimental work on animals was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Science and with approval of the local animal experimentation authorities (Az 11-0637, LAVES). Apolipoprotein E deficient mice, 8 weeks of age (Jackson Laboratories, Bar Harbor, ME) were fed a Western diet containing 1.25% cholesterol for 7 weeks or 10 weeks.

After 7 or 10 weeks of Western diet mice were anesthetized using 1.5Vol% Isoflurane in 100% oxygen. The surgical field was shaved. In the double hit group, mice were subjected to a median laparotomy that was left open for 30 minutes. Also, they were subjected to a blood loss from the retrobulbar plexus of roughly 20% (400µl) based on the assumption that blood volume of small rodents is 7.3% of their body weight. The laparotomy wound was closed by single-knot sutures. 72 hours after the double hit mice were reanesthetized and sacrificed by drawing total blood from the caval vein. Mice were perfused with 0.9% saline at 90mmHg pressure from a free flowing bag through the left ventricular apex. Blood was drained from a right atriotomy until saline was flowing clear. Sham animals experienced 30 minutes of general anesthesia and their abdomen was shaved without any further intervention. Sham animals were also sacrificed after 72 hours the same way as were mice in the double hit group.

In the metoprolol treatment group mice received metoprolol (aiming for a dose of 250mg/kg body weight/d p.o.) or propranolol (aiming for a dose of 30mg/kg body weight/d p.o.) dissolved in the drinking water starting 3 days before surgery until 3 days post surgery. Metoprolol or propranolol were dissolved in the drinking water at a concentration of 1.8mg/ml or 0.225mg/ml. With their drinking water mice took in the daily dose of drug. Volume of water use (4
ml/d*mouse) was monitored and did not significantly decline with start of drug dosing. Blood pressure and heart rate were assessed in metoprolol treated animals before the start of the metoprolol treatment and at the time of sacrifice using a tail cuff device in isoflurane anesthesia. The innominate artery was dissected and embedded in Optimal Cutting Temperature-compound for histology and immunohistochemistry. The aorta was dissected from the sinus to the bifurcation and embedded (thoracic) or snap frozen (abdominal) in liquid nitrogen.

2.3 Plasma lipid profile, histology and plaque size measurements

Plasma samples were collected from all animals. Total cholesterol, triglycerides (Roche Molecular Biochemicals, Mannheim, Germany) and phospholipid (Wako, Neuss, Germany) levels were determined enzymatically using commercially available kits. The assessment of cholesterol content in lipoprotein subclasses was determined following sequential tabletop ultracentrifugation as published.96 Cholesterol concentrations in individual fractions were then measured as described above.

The innominate artery was cryosectioned at 7µm thickness and stained. Plaque size, quantified on hematoxylin and eosin (H&E) staining, was assessed on the ten sections of the innominate artery immediately distal of the bifurcation 49 µm apart from each other spanning the proximal 500 µm of the vessel. Internal elastic lamina (IEL) and lumen area were morphometrically assessed using Cell-F life science fluorescence imaging software version 3.1 and plaque size was calculated as IEL-lumen size. Plaque size was calculated as average of the 10 assessed sections. Animals with sections showing cutting artifacts were excluded
from the analysis.

**H&E Staining Protocol**

1. Warm the sections for 30 min at RT
2. Stain in Mayer hematoxylin solution for 5 min
3. Wash in warm running tap water for 5 min
4. Counter stain in eosin Y solution for 3 min
5. Wash in warm running tap water for 3 min
6. Dehydrate twice through 70% alcohol 1 min each, 2 changes of 100% ethanol 5 min each
7. Clear in 2 changes of xylene, 5 min each
8. Mount with corbit-balsam

**2.4 Plaque composition and stability as assessed by immunofluorescence and immunohistochemistry**

Immediately adjacent sections were stained for macrophages (rat anti-mouse CD68, clone FA-11, 1:100), vascular smooth muscle cells (mouse anti-human/mouse α-smooth muscle actin; αSMA, clone 1A4, 1:100) or TIMP-2 (goat anti-human/mouse AF971, 1:100). For detection Cy3-coupled secondary antibodies (goat anti-rat or goat anti-mouse, 1:100) were used and slides were mounted with fluorescent mounting medium (S3023, DAKO). Micrographs were captured using Q-capture, a fluorescence imaging software driving a Retiga CCD camera mounted on an Olympus IX81 microscope (Olympus Europa Holding GmbH, Hamburg, Germany). Morphometry was performed using Cell-F life science fluorescence imaging software version 3.1. Macrophage, VSMC and
TIMP-2 content were quantified as the percentage of the lesion staining positive for macrophage-specific marker CD68, VSMC marker αSMA or TIMP-2.

Immunofluorescence Staining Protocol

1. Warm the sections for 30 min at RT
2. Fix the sections in cold acetone (-20 °C) for 10 min and dry for 20 min at RT
3. Wash in PBS/Tween-0.05% for 5 min.
4. Block sections with M.O.M blocking reagent, 1%BSA/PBS for 30 min where needed (VSMC)
5. Incubate sections with primary antibodies (diluted with 1%BSA to 1:100) overnight
6. Rinse with PBS 3 times for 5 min each
7. Incubate sections for 2 h in FITC-labeled secondary antibody (diluted with 1% BSA to 1:100) in 37°C and rinse with PBS once for 5 min
8. Counter stain with DAPI (diluted with PBS to a final concentration of 1:10000) for 5 min
9. Rinse with PBS 3 times for 5 min each and wash with distilled water once
10. Add 1-2 drops of water mounting medium and cover with glass cover slip.

2.5 Morphology score

To assess plaque complexity and vulnerability, plaques were scored following the Stary criteria. Since the Stary score collects complex plaques in only few categories, we additionally developed a novel score including three features of the Stary score: necrosis, hemorrhage and buried fibrous caps that can indicate plaque complexity. One point was assigned for every feature present. Lesions
were scored as presenting with necrotic core when a hematoxylin free area containing debris or lipids was observed in the center or at the base of the plaque. Cell free areas can be due to cutting artifacts. To avoid misinterpretation, lesions were only considered to have a necrotic core if at least 50% of the examined sections presented a core necrosis. Intraplaque hemorrhage was defined as clusters of erythrocytes present in the plaque interior of at least 10% of the sections on H&E stainings and if in addition red blood cell autofluorescence in the FITC channel was positive. Buried fibrous caps caused by plaque cap rupture and overgrowth of new plaque material were detected as clusters of cap-like organized VSMC within the plaque on αSMA-immunohistochemical stainings that were covered by newly formed plaque on any of the analyzed sections stained for αSMA. Each of the three features was assessed on H&E and αSMA-stainings. For every feature one scoring point was assigned. The scoring points were added to calculate the score. Ten sections per animal were used for scoring plaque complexity. Data are presented as the percentage of each score present within the group. Mice without plaque were assigned a score of 0.

2.6 Quantitative real-time PCR (qRT-PCR)

Aortic tissues and cultured cells were harvested and total RNA was isolated with TRItidy reagent (Applichem, Darmstadt, Germany). cDNA was prepared using random nanomers, and qRT-PCR was performed with primer for TIMP-2. HPRT was used as housekeeper. The sequences of the PCR primer were as follows: TIMP-2, 5'-TGACATCCCTTCCTGGAAACAGCA-3' (forward) and 5'-ACTCCTGCCTGTAGCAAGGATCAA-3' (reverse); HPRT, 5'-
TGATCAGTCAACGGGGGACATA-3’ (forward) and 5’-
GCCTGTATCCAACACTTCGAGA-3’ (reverse). All samples were run in duplicates
on a RT-PCR cycler (Rotogene 3000, Corbett Life Science, Hilden, Germany) using
SYBR-GREEN (SensiFAST TM SYBR, Bioline GmbH, Germany) and normalized to
HPRT gene expression. Data are expressed as $2^{-\Delta(\Delta CT)}$.

RNA extraction protocol

1. Homogenize aorta with pestle homogenizer and resuspend in 500µl
TRIzol
2. Heat the samples for 5 min at 28 °C
3. Add 100µl chloroform to each sample and invert for 15 sec
4. Heat the samples for 3min at 28 °C
5. Centrifuge the samples for 15 min at 13,000 rpm at 4 °C
6. Transfer the upper aqueous phase to a new tube, add 250µl 2-propanol per
sample and incubate for 10 min at 28 °C
7. Centrifuge again for 10 min at 13,000 rpm at 4 °C, remove the supernatant
and wash RNA once with 500µl 75% ethanol
8. Centrifuge for 10 min at 10,000 rpm at 4 °C, discard the supernatant and
dissolve RNA in RNAse-free water
9. Incubate 10 min at 60 °C and keep it on ice
10. Measure the concentration by photometer for absorbance at 260 nm

cDNA synthesis protocol

500ng of total RNA were mixed with master mix containing 1µl 10X reaction
buffer, 2µl 25mM MgCl$_2$, 2µl 2.5mM deoxynucleotide triphosphates (dNTP),
0.25µl OligodT primer, 0.25µl random nonamer, 0.25µl RNase Inhibitor, 0.25µl Euroscript RT and RNase free water for a total volume of 10 µl. Mixed components were centrifuged and incubated in a Biometra Cycler at 25 °C for 10 min, 48 °C for 30 min. The reaction was stopped by heating to 95 °C for 5 min. Diluted cDNA in RNAse free water at a 1:1 ratio and 0.5µl of reverse transcription reaction products were used for qRT-PCR.

2.7 Zymography to assess gelatinolytic activity

Aorta samples were homogenized with homogenization buffer (50mM Tris base, 0.2% Triton in ddH₂O) and the MMP containing supernatants were measured by BCA to assure loading of equal amounts of samples on Novex 10% gelatine zymogram gels. Zymography was performed following the protocol described previously. Photographs were taken and band intensities were measured with ImageJ 1.48v analysis software.

Zymography assay protocol

1. Mix an equal amount of sample and gel-loading buffer, load into the wells of the gel
2. Run the gel at 125 V constant for 90 minutes
3. Place the gel in to 100ml renaturing buffer and incubate for 30 min at RT with gentle agitation
4. Remove the renaturing buffer and add 100 ml developing buffer, incubate for 30 min at RT with gentle agitation
5. Change 100ml developing buffer and incubate overnight at 37 °C with gentle agitation
6. Wash the gel 3 times with deionized water at RT with gentle agitation, 5 min each time

7. Stain the gel with 20 ml Coomassie blue for 1 hour at RT with gentle agitation

8. Destain the gel with 100 ml deionized water for 1 hour at RT with gentle agitation

9. Scan the gel and take photographs

2.8 Cell culture

Macrovascular human endothelial cells were purchased (Promocell, Germany), macrophages were differentiated from THP-1 cells incubated with phorbol myristate acetate (PMA) 100ng/ml for 48h and VSMC were isolated from human radial arteries from graft leftovers with a waiver from the ethical committee. Cells were seeded in 6-well plates with 2.5 x 10^5 per well and cultured with 5%EGM-2, 10%RPMI 1640 and 20%DMEM until 100% confluent. Subsequently, cells were incubated overnight in serum free medium for starvation. Cells were then treated with epinephrine (10µM), oxLDL (20µg/ml) and/or metoprolol (100nM). Dose response curves revealed these as the highest concentrations of the agonists with no toxicity as assessed by viability using trypan blue exclusion. All cultured cells were incubated at 37°C under a humidified atmosphere containing 5% CO2. All experiments were performed using cell passages 4-10. Culture supernatants were removed after 24 hours, the cells were washed twice with PBS, collected in TRl tidy and frozen at -80°C until assayed.

2.9 Statistical analysis

Data are presented as median and 5%/95% percentiles in whisker blots. Data
had non-Gaussian distributions or significantly different standard deviations between groups unless otherwise noted. Kruskal-Wallis test was employed for global assessment of differences in the data family followed by Mann–Whitney U test for group wise comparisons (Prism 5, GraphPad Prism). Fisher’s exact test was used to compare incidences of plaque features in the stability score studies. Multiple testing was accounted for by holms correction. P values less than 0.05 were considered significant. Linear regression analysis was used to determine the direction and extent of correlations between the two variables.
3. Results

3.1 Lipoprotein profiles

We measured plasma lipoprotein concentrations to assure that the diet induced hypercholesterolemia and to observe if interventions exerted effects on lipoprotein levels. The diet induced stable hypercholesterolemia in all animals. The effects of the double hit and its components on lipoprotein profiles have been described elsewhere. None of the alterations reached statistically significant levels.

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides (mg/ml)</th>
<th>total chol. (mg/ml)</th>
<th>VLDL-chol. (mg/ml)</th>
<th>LDL-chol. (mg/ml)</th>
<th>HDL-chol. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (n=9)</td>
<td>90±39</td>
<td>547±149</td>
<td>369±139</td>
<td>130±15</td>
<td>48±8</td>
</tr>
<tr>
<td>Sham (n=11)</td>
<td>78±21</td>
<td>519±134</td>
<td>324±102</td>
<td>146±30</td>
<td>50±10</td>
</tr>
<tr>
<td>Double hit (n=19)</td>
<td>68±23</td>
<td>426±91</td>
<td>263±72</td>
<td>119±21</td>
<td>45±8</td>
</tr>
<tr>
<td>DH &amp; metop (n=25)</td>
<td>58±17</td>
<td>393±95</td>
<td>231±68</td>
<td>119±28</td>
<td>43±10</td>
</tr>
</tbody>
</table>

Table 2: Lipoprotein profiles demonstrate a robust hypercholesterolemia that does not significantly change with the chosen intervention of the double hit. Values from normally distributed data families are presented as means ± standard deviation. Group sizes are somewhat different because the double hit and treatment animals were stratified in different subgroups for plaque analysis.

3.2. Hemodynamic response to β-adrenoceptor blockade

We intended to examine the effects of blood pressure and heart rate lowering on plaque size and stability. After administration of metoprolol, heart rate of mice decreased slightly if at all comparing baseline to the time point immediately prior to surgery. Mice largely regulate their cardiac output demand by adapting heart rate. BRB effects on heart rate are thus less pronounced than on blood pressure. (Figure 3) Blood pressure decreased in about 50% of the animals comparing baseline prior to metoprolol treatment to the time pre surgery. (Table 3) An increase in the metoprolol dose did not result in further reductions in
heart rate or blood pressure (data not shown). Mice were therefore stratified into responders to metoprolol therapy when blood pressure decreased by more than 10% at the time of surgery and non-responders if blood pressure did not decrease by more than 10%.

Figure 3: Hemodynamics at baseline and pre surgery in mice on diet for 7 or 10 weeks. Mouse drinking water intake was monitored and did not decrease with the addition of metoprolol. The drug was thus taken up by the animals. The hemodynamic effect was however not uniform. Heart rate responses were overall weak. Blood pressure did decrease in about 50% of the animals. Since we wanted to test the hypothesis that metoprolol-mediated decreases in hemodynamic strain would affect plaque size and stability, we defined efficient metoprolol treatment as a decrease >10% in mean arterial pressure comparing baseline values and values at the time of the procedure. Animals were therefore separately analyzed as responders and non-responders with respect to plaque size and vulnerability.

Mice were stratified into responders to metoprolol therapy if blood pressure decreased by more than 10% at the time of surgery and otherwise as non-responders. Non-responders and responders were analyzed separately with respect to the development of plaques and their complexity.
Table 3: Blood pressure at baseline and pre surgery in responders. Metoprolol administration decreased blood pressure in these animals comparing baseline to pre surgery. (*=p<0.05 vs baseline)

3.3 Development of plaque size

If attenuation of hemodynamic stress affects plaque load or complexity has not been addressed previously. The response of plaque development and progression after the double hit was therefore tested in mice sacrificed after 7 or 10 weeks of diet and compared to animals with decreased blood pressure in response to metoprolol treatment. Mice on the diet for 7 weeks hardly presented plaques in the sham group. This group was used to examine if new plaque formation would occur in response to the double hit and if it would be amenable to metoprolol treatment. The double hit induced rapid and significant plaque growth in mice that were on the diet for 7 weeks. (Figure 4A) Plaque growth was significantly reduced in mice with lower blood pressure after metoprolol treatment. 10 weeks of diet induced larger plaques at baseline. This condition was therefore suited to examine if the double hit would increase and metoprolol would reduce the size of preexisting plaques. Here, the double hit did not further increase plaque size. Blood pressure lowering with metoprolol did also not affect plaque load. (Figure 4B) No significant differences were observed between the group of animals that mounted a hemodynamic response to metoprolol and those that did not in either condition.
Figure 4: Plaque size in Apo-E deficient mice after 7 and 10 weeks of high cholesterol diet.
A) The double hit induced a significant growth in plaque size in the mice on a Western diet for 7 weeks compared to sham mice. Metoprolol induced a significant prevention of plaque growth in these animals. B) In mice, which were on the diet for 10 weeks, plaques at the branching point had developed at the start of the experiment. These more advanced plaques did not grow further in response to stress. Plaque size was not significantly affected by the double hit or by blood pressure lowering by metoprolol treatment. There was no significant difference in plaque size between responders and non-responders.
3.4 Morphology score in mice exposed to perioperative stress

We next questioned if perioperative stress would induce growth of more unstable plaques or destabilize existing plaques in the two conditions. The Stary score is commonly used for classification of plaque stability in human atherosclerosis grouping lesions to six plaque types with increasing plaque complexity.97 Double hit caused a significant increase in the proportion of complex plaques in category IV, V and VI in animals placed on the diet for 7 weeks compared to the sham group. Metoprolol-treatment with blood pressure reduction reduced the proportion of complex plaques as assessed by the Stary score. (Table 4) In animals on the diet for 10 weeks plaques with considerable complexity had developed spontaneously and the double hit only had a slight, non-significant effect to induce signs of plaque vulnerability. The Stary score does, however, not provide a sufficiently high resolution of differences in vulnerability, since it collects all complex plaques (ruptured and rupture prone) in class VI where surface defects, thrombosis and hemorrhage are grouped together. Therefore, we additionally scored the individual components of the Stary score by a novel score as described previously,77 in which the most stable plaque would have no necrosis, hemorrhage or buried fibrous caps, while the most vulnerable plaques would exhibit all three features and score three points. In mice, 7 weeks on diet, high plaque scores (2 or 3 points), reflecting significantly more complex, newly developed plaques, were more frequent in animals subjected to the double hit compared to the sham group. Sham animals in turn had more frequently low plaque scores (0 or 1 point).
Table 4: Scoring of plaque complexity using the Stary score. Plaque complexity in the groups sham, double hit and double hit plus metoprolol are presented as percentages. In animals on the diet for 7 weeks, the sham group had mostly no or very small plaques. Double hit caused a significant increase in the incidence of complex plaques category IV, V and VI. However, the proportion of mice presenting with complex categories was significantly decreased after metoprolol treatment. In animals on the diet for 10 weeks the double hit did not change the complexity of the plaques. Metoprolol treatment, however, reduced the complexity of previously established plaques. (Chi square test: *=p<0.05 vs. sham, #=p<0.05 vs. double hit)

<table>
<thead>
<tr>
<th>Stary Score in 7 weeks diet group</th>
<th>Sham (%)</th>
<th>Double hit (%) *</th>
<th>Double hit + Metoprolol (%) #</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Isolated macrophage foam cells</td>
<td>43</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>II Multiple foam cell layers</td>
<td>14</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>III Isolated extracellular lipid pools</td>
<td>43</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>IV Confluent extracellular lipid core formed</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>V Fibromuscular tissue layers produced</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>VI Surface defect, hematoma, thrombosis</td>
<td>0</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stary Score in 10 weeks diet group</th>
<th>Sham (%)</th>
<th>Double hit (%)</th>
<th>Double hit + Metoprolol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Isolated macrophage foam cells</td>
<td>8</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>II Multiple foam cell layers</td>
<td>15</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>III Isolated extracellular lipid pools</td>
<td>0</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>IV Confluent extracellular lipid core formed</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>V Fibromuscular tissue layers produced</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VI Surface defect, hematoma, thrombosis</td>
<td>62</td>
<td>67</td>
<td>58</td>
</tr>
</tbody>
</table>

Figure 5: Morphology score of plaque complexity. A) In mice on the diet for 7 weeks, there were less high scores (2 or 3 points) in sham mice and the number of high scores increased to more than 60% in the double hit group. The number of mice scoring 2 or 3 points fell to 20% after metoprolol treatment. B) In 10 weeks diet mice, no alteration in plaque complexity was seen after the double hit. Metoprolol treatment reduced the number of mice scoring 2 or 3 points. Metoprolol treatment with blood pressure reduction significantly reduced the proportion of plaques with a high score in the double hit group (Figure 5A).
Plaques were already much more complex after 10 weeks of diet in the sham group. The double hit had no apparent effect on the complexity of the preexisting plaques, and therefore they had similar scores as the sham group. Metoprolol treatment however mildly reduced the complexity of previously established plaques. (Figure 5B)

3.5 Plaque composition in mice exposed to the double hit

To assess if plaque composition changed due to the double hit or by metoprolol treatment, we examined the content of macrophages and vascular smooth muscle cells in the lesion area. CD68 and αSMA are markers for macrophages and VSMC respectively that are used to assess plaque stability. CD68 content of the plaque did not significantly change due to the double hit both in mice on the diet for 7 or 10 weeks compared to sham animals. (Figure 6) αSMA content of the plaque in the double hit group was slightly altered compared to sham animals. It significantly increased by metoprolol treatment in mice on the diet for 7 weeks but not in animals fed for 10 weeks. (Figure 7) In summary, we did not detect a meaningful alteration in plaque composition after metoprolol treatment that reduced blood pressure.
Figure 6: Macrophage content of plaques after double hit and metoprolol. A) There were no differences of CD68 expression in the plaque among sham group, double hit group and metoprolol treatment group in mice fed a diet for 7 weeks. B) In mice fed the diet for 10 weeks, CD68 content in the plaque increased slightly but non significantly in the double hit group compared to the sham group: Likewise the trend for a decrease after treatment with metoprolol was not statistically significant. No difference was noted between metoprolol responders and non-responders. Typical examples of microphotographs are shown below the whisker blots for all experimental groups. Bar indicates 100µm.
Figure 7: VSMC content of the plaques after double hit and metoprolol treatment. A) αSMA content did not change between sham group and double hit in mice on the diet for 7 weeks. However, metoprolol treatment increased αSMA content in plaque significantly compared to the double hit group. B) There was no difference of αSMA content between sham and double hit in mice on the diet for 10 weeks. Metoprolol treatment in animals with established lesions only slightly increased αSMA content in plaque compared to the double hit group with no treatment. No differences were observed in mice that exhibited a blood pressure lowering effect versus the non responders. Bar indicates 100µm.
3.6 Effects of hemodynamic changes and propranolol effects on plaque size and stability

We observed a reduced plaque growth response in mice with small lesions and reduced plaque complexity induced by perioperative stress in newly grown and existing lesions. A metoprolol-induced blood pressure reduction had, however, no measurable effect on plaque composition. The reduction of plaque growth and the increase in stability could be due to the hemodynamic effects of metoprolol or could be due to pleiotropic effects of metoprolol. We therefore addressed the question if hemodynamic alterations were associated with alterations in plaque growth or stability by comparing the mice that had not responded to metoprolol to those that had.

All animals that had been treated with metoprolol were examined in correlation analyses to establish if hemodynamic effects correlated with plaque size or complexity. There was no linear correlation between plaque area with the changes of heart rate and blood pressure in response to metoprolol treatment. (Figure 8A, B) Since the stability score as a categorical parameter in nature is not accessible to correlation analysis, we compared the changes in hemodynamic parameters between animals grouped according to the complexity scores. If complexity would be driven by hemodynamic impact differences should be detectable (Figure 8C, D, E, F) No differences in the magnitude of hemodynamic changes were observable.
Figure 8: Effect of metoprolol on plaque size does not correlate with hemodynamic alterations. A, B) No significant correlation of plaque size with the changes of HR and BP was observed in either group. C, D, E, F) Hemodynamic responses in mice on Western diet for 7 or 10 weeks were not different in animals presenting with increasing complexity scores, although this analysis has to be interpreted with caution.

To assure that the observed effects were due to the BRB effect we asked if propranolol had comparable effects on plaque size and complexity as metoprolol by treating mice undergoing the double hit model after 10 weeks of diet. Similar to metoprolol did propranolol-mediated blood pressure reduction have no effect on plaque growth when mice experienced the double hit. (Figure 9A) In addition
was plaque size not different between responders and non-responders to propranolol treatment in mice on the diet after 10 weeks, (Figure 9B)

![Graph showing plaque size and morphology score in propranolol treated mice.]

Figure 9: Plaque size and morphology score in propranolol treated mice. A) Plaque area was not significantly affected by blood pressure lowering after propranolol treatment, B) Between non-responders and responders, plaque size was not significantly different. C) Propranolol treatment reduced the number of mice scoring 2 or 3 points in the novel complexity score.

<table>
<thead>
<tr>
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<th>Double hit (%)</th>
<th>Double hit + Propranolol (%)</th>
</tr>
</thead>
<tbody>
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<td>28</td>
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<tr>
<td>VI Surface defect, hematoma, thrombosis</td>
<td>62</td>
<td>67</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 5: Stary score for propranolol treatment mice. Propranolol reduced the incidence of complex plaques categories compared to double hit group.
Since metoprolol reduced plaque complexity in these mice, we assessed plaque complexity by Stary scoring as well as scoring following our novel score. (Table 5, Figure 9C) The proportion of plaques in category IV, V and VI was reduced in animals after propranolol treatment as it was for metoprolol.

3.7 Effect of metoprolol treatment on TIMP-2 expression in murine aortas from double hit animals

Since BRB had an effect on plaque growth and on signs of vulnerability but neither hemodynamic effects nor choice of substance seemed to cause the effect, we reasoned that features of the vascular wall microenvironment could be responsible. We therefore searched for pleiotropic effects of metoprolol in our model and in vitro models of vascular wall primary cells.

As layed out above, plaque stability is controlled by the family of matrix metalloproteinases and their tissue inhibitors. TIMP-2 opposes proteolytic activity of MMPs in destabilization of atherosclerotic plaques and is therefore thought to be a factor that could induce stabilization of plaques.\textsuperscript{85} TIMP-2 expression is increased by BRB in myocardium after infarction.\textsuperscript{100} To investigate potential changes of the expression of TIMP-2 after metoprolol treatment, real time PCR, zymography assays and anti-TIMP-2 immunohistochemistry stainings were performed. qRT-PCR from aortic tissue from the mice in our model demonstrated a significant increase in TIMP-2 expression when mice were treated with metoprolol before the double hit (Figure 10A). We also observed a decrease in MMP-9-associated gelatinolytic activity in the metoprolol treated double hit group compared to the double hit group without treatment in zymographies. (Figure 10B) Using immunofluorescence we in parallel detected
an increase in TIMP-2 signal in lesions when animals were treated with metoprolol, compared to the double hit group. (Figure 10C)

Figure 10: TIMP-2 expression and activity increased in mice exposed to double hit and treated with metoprolol compared to untreated animals. A) TIMP-2 mRNA expression in aorta was significantly increased in the metoprolol treated compared to the untreated animals. B) To estimate MMP-9-associated proteolytic activity, aortic tissues were tested by zymography. MMP-9-associated proteolytic activity was decreased in double hit animals treated with metoprolol compared to the untreated animals. C) Sections of the innominate arteries were stained for TIMP-2 and CD68. DAPI was used as a nuclear staining. The percentage of plaque material staining positive for TIMP-2 (red) was significantly increased by metoprolol compared to untreated animals. TIMP-2-expression was observed in macrophages and in the endothelial lining. Bar indicates 100µm.
3.8 Effect of metoprolol on TIMP-2 expression in macrophages, VSMC and endothelial cells

We next asked if metoprolol exerted its effect on specific cell types that typically constitute the vascular wall. TIMP2 expression was therefore studied in macrophages, endothelial cells and VSMC that were subjected to oxLDL stimulation and co-incubated with epinephrine in the presence or absence of metoprolol.

**Figure 11: TIMP2 mRNA expression in macrophages, endothelial cells and VSMC.** Human macrophages, primary human microvascular endothelial cells and human VSMC were cultured with oxLDL, epinephrine and metoprolol in numerous conditions and concentrations. Cells were harvested and TIMP2 expression at mRNA level was assessed. Depicted is the longest incubation with the individual highest concentrations of oxLDL (20µg/ml), epinephrine (10µM) and metoprolol (100nM). No effects on individual cell lines were detectable in any of the tested conditions.
However, TIMP2 expression in isolated cells was not significantly altered by oxLDL, epinephrine or metoprolol alone or in combination in all three cell types under a variety of different conditions and with increasing concentrations. Figure 11 depicted a single condition and concentration study as a segment of this large body of results.
4. Discussion

Patients bearing vulnerable plaques and undergoing major surgical procedures are at high risk for plaque rupture and ensuing infarction due to perioperative stress. PMI occurs in about 400,000 patients in Europe each year and is associated with a high mortality.\textsuperscript{37,61,101,102} The particular mechanisms of plaque rupture in association with surgical procedures and perioperative stress are largely unknown.\textsuperscript{39,101,103} The conventional understanding of perioperative plaque rupture focuses on altered hemodynamics in the perioperative period. Part of this conception is a chronic, progressive plaque that is not subjected to rapid modification. However, we recently demonstrated in a mouse model of perioperative stress that lesion size and the plaques’ composition that is responsible for plaque stability is much more dynamic than previously thought. We reported that perioperative stress rapidly increases plaque size and affects its vulnerability.

In clinical routine only limited strategies are available to prevent PMI. The cardio-protective potential of statins and BRB for protection of high-risk patients undergoing major surgical procedures has been tested in several studies.\textsuperscript{58-60} Despite the controversial discussion of their overall benefits both have been found to effectively reduce the rate of PMI in man.\textsuperscript{104-106} For both strategies, the underlying mechanism has not been explored in detail. For BRB, authors believe that they limit myocardial oxygen consumption by preventing heart rate increase and reducing myocardial contractility and thus restore the oxygen supply-demand mismatch.\textsuperscript{37,62} However, direct anti-atherosclerotic effects of long term BRB use have been described in animal experiments\textsuperscript{66-68} as well as clinical studies.\textsuperscript{63-65} If BRB prevent rapid plaque destabilization in the perioperative
setting was the subject of this investigation.

Johnson and coworkers have originally reported that plaque rupture specifically occurs in the innominate artery of ApoE\(^{-/-}\) mice after eight weeks of Western diet.\(^{76}\) We set up a double hit model of postoperative plaque destabilization mimicking perioperative stress by the combination of a laparotomy with acute non-replaced loss of 20% blood volume in ApoE\(^{-/-}\) mice based on the observations of Johnson.\(^{77}\) We demonstrated that the double hit of surgery and blood loss can drive plaque growth and induce more complex lesions compared to respective controls. The effects of this double hit could be ameliorated by statins and IL-6 blockade.

We used this novel model in the present study to explore BRB effects. In ApoE\(^{-/-}\) mice exposed to the double hit of surgery and blood loss atherosclerotic plaque growth and complexity were reduced if animals were treated with a high dose, short-term metoprolol regimen. In mice fed a Western diet for 10 weeks leading to advanced plaques at the time of the double hit, existing plaque size was not affected by metoprolol treatment while plaque stability was significantly improved. The VSMC and macrophage content of the plaques was largely unaffected by metoprolol treatment with a trend for an increase in VSMC positive area with metoprolol treatment. Interestingly, and contradicting the general view in the existing literature, was plaque load or stability not associated with changes in hemodynamic parameters in our study. At the same time were hemodynamic effects of the metoprolol treatment mild. On the contrary was TIMP-2-protein and activity increased in the diseased vessel wall and MMP-9-associated proteolytic activity decreased in animals treated with metoprolol. This effect of metoprolol on TIMP-2 expression and activity was not present in
human primary cells in vitro.

In a second group mice were fed the atherogenic diet for 7 weeks. In these mice, prone for atherosclerosis, but with nearly absent plaque development at the end of week 7, the double hit induced plaque growth. The double hit also caused a significant increase in plaque complexity. Both adverse effects were reduced significantly by metoprolol treatment. Johnson and colleagues have reported that complex lesions with spontaneous disruption of the luminal plaque surface and growth of plaque material over these areas develop in the innominate artery during week eight of Western diet in Apo-E knockout mice. We speculated that perioperative stress during week eight would amplify and accelerate the rate of plaque rupture. In our study, we observed the expected results. Mice on the diet for 7 weeks presented small or no plaques (Baseline). Mice fed for another 3 days (Sham) had likewise small or no plaques. In this regard were our mice somewhat slower in plaque development compared to the mice that Johnson and colleagues used with the exact same diet. This phenomenon is well known and susceptibility of animals to plaque development is subject to subtle influences and considerable variability from laboratory to laboratory. Our ApoE\textsuperscript{−/−} animals were backcrossed into C57B6/J animals for more than ten generations. The diet chosen was commercially mixed according to the description from Johnson and colleagues. We therefore believed that plaque development was simply somewhat delayed in our animals. This notion finds supported in the observation that after 10 weeks of diet plaques in our animals were already large and complex in Sham and Baseline groups, suggesting that week 9 in our study likely corresponds to the week that Johnson and colleagues reported as week 8 in their colony.
The double hit at the early time point induced growth of plaques with measurable complexity. This de novo plaque growth was amenable to a high dose, short-term metoprolol treatment. Metoprolol as a classic cardioselective BRB blocks the effects of the catecholamines norepinephrine and epinephrine on β1-adrenergic receptors, thus decreasing the effects of the sympathetic nervous system on cardiomyocytes. This results in lower heart rate and reduced blood pressure in humans, which in aggregate reduces myocardial oxygen consumption and also reduces coronary blood flow and thereby shear stress on the vascular surface due to prolongation of the diastoly.

Early data from the eighties suggest that metoprolol would also negatively affect lipid metabolism by increasing the level of low-density lipoprotein and lowering the level of high-density lipoprotein. We did not observe such effects in our animals. We rather saw a decrease of total cholesterol in the metoprolol treated groups. Inflammation and stress have also been reported to cause alterations in lipoprotein levels. In our study, neither the double hit nor metoprolol treatment had a meaningful effect on atherogenic lipoprotein profiles. The lowering of the atherogenic lipoproteins VLDL and LDL has also been reported in our recently published paper. We cannot explain this observation, but the fact that plaques grow in the presence of reduced atherogenic pressure suggests that these alterations in lipoprotein concentrations are not biologically relevant.

β-adrenoeceptor antagonists have been used for treatment of cardiovascular diseases for many years. They have become indispensable in the treatment of ischemic heart disease. Several clinical trials have proven antiatherosclerotic effects of long-term treatment with metoprolol. In the beta-blocker
cholesterol-lowering asymptomatic plaque study (BCAPS), plaques are more echogenic in participants treated with metoprolol for 36 month than in those not treated with metoprolol. Gray scale median on ultrasound images have increased significantly more in the metoprolol treated subjects. And metoprolol could also reduce the rate of progression of carotid intima-media thickness in clinically healthy, symptom-free subjects with carotid plaque. More recently, the large Perioperative Ischemic Evaluation (POISE) trial reports a reduction of nonfatal PMI by 26% in patients undergoing non-cardiac surgery, who are treated with a rather high dose of metoprolol. This dose reduces heart rate and blood pressure in some individuals, which causes stroke rates and fatalities to increase, while the rate of MI decreased as had been predicted.\textsuperscript{110}

Our data support this observation from the POISE and other trials, because we found that plaque growth and complexity were significantly reduced in mice suffering double hit with a high dose, short-term metoprolol treatment. However, numerous clinical trials have examined the effect of BRB on perioperative cardiovascular complications with very divergent results. While BRB effects on mortality are contradictory, most trials reveal a well detectable effect on the rate of PMI.\textsuperscript{111-113} We suggest that one important effect of BRB during the perioperative phase may reside in its ability to prevent de novo plaque formation.

We further asked if advanced plaques were affected by double hit or metoprolol treatment. If plaque stability and thereby vulnerability to rupture can be subject to change due to perioperative stress is currently unknown.\textsuperscript{114,115} We used the previously published vulnerability score, in order to assess the plaque complexity.\textsuperscript{77} Compared with the Stary criteria, this score discerns necrosis,
hemorrhage and buried fibrous caps into individual items allowing for a more detailed assessment of plaque complexity. Mice subjected to the double hit after 7 weeks of Western diet rapidly developed plaques with signs of vulnerability and metoprolol could prevent this. Interestingly, the double hit did not increase the complexity of preexisting plaques in the mice placed on the Western diet for 10 weeks. Metoprolol treatment however also promoted a small but significant reduction of the complexity of previously established plaques. These results suggested that metoprolol was efficient in preventing newly developed plaque and to prevent this plaque to be primarily vulnerable. It also reduced but to a much lesser extent the vulnerability of previously present plaques.

Other studies likewise report that atherosclerotic plaques can undergo very rapid changes in volume, composition and phenotype. BRB according to our data had a slim effect on existing plaque alterations under the conditions studied here, but a rather pronounced effect on new plaque formation due to perioperative stress. One avenue that could account for differences in characteristics of newly formed plaques could be effects on plaque composition. In Johnson’s study, it is suggested that the observed atherosclerotic plaque growth and destabilization was due to modulation of macrophages and foam cell behavior. Macrophage content can change rapidly in plaques. Shah and coworkers report that within only 48 hours after apoA-I Milano administration about 50% of the lipids and macrophages have been eliminated from the lesions in ApoE−/− mice. Llodra and colleagues go on to demonstrate that if established plaques are transplanted into recipient mice with the same atherogenic phenotype, 50% of the macrophages are exchanged within 72 hours, and if plaques are transplanted into animals without atherogenic diet and disposition.
50% of the macrophages have left the lesion after only 3 days. These data suggest a very active recirculation of macrophages and very fast dynamic of the composition of atherosclerotic lesions. This notion has however also been challenged and the major role is shown to reside in a reduction of macrophage recruitment into the plaque at least during disease regression. This could suggest that a preference for recruitment of macrophages would expand lesions by increasing the relative contribution of macrophages or foam cells to plaque load. However in our study we did not observe significant changes of macrophage content in the short-term intervention for acute plaque instability both in the mice on the diet for 7 or 10 weeks.

The content of VSMC is another determinant of the stability of plaques. The association of VSMC content and plaque stability has been shown in numerous studies. We here showed that αSMA content of the plaque was unchanged after metoprolol treatment. Relative macrophage and vascular smooth muscle content did not change significantly, suggesting that a net increase of the recruitment of both cell types occurred during the 72 hours following the insult. Our study falls short of a full characterization of plaque composition and therefore it remains possible that other constituents of the plaque changed in response to the metoprolol treatment, which could have driven the increase in signs of vulnerability. An important constituent of plaques is the extracellular matrix and here collagen content is a major driver of plaque stability. Collagen content as such is however not as central to the plaque stability as its structural integrity. It is to date unclear how fast extracellular matrix or cellular components of plaque and thereby their stability or vulnerability to rupture can be altered.
Hemodynamic alterations secondary to surgical procedures and the resulting mechanical strain on the vessel wall has been held responsible for precipitation of plaque rupture after surgery. Hypertension is a well-known risk factor for atherosclerosis in humans. Tachycardia is associated with a greatly increased risk of plaque rupture in patients with coronary atherosclerosis. Elevated heart rate caused by mechanical stress may promote weakening of the fibrous cap, ultimately increasing the risk of plaque disruption. Clinical trials suggest that heart rate reduction is an important component of the benefits of BRB in stable angina pectoris, after MI and in heart failure. Most authors claim that the cardio- or vasculoprotective effect of metoprolol resides in the lowering of heart rate and myocardial contractility which reduces shear stress and myocardial oxygen demand at the same time. However, metoprolol has mild effect on blood pressure in normotensive patients and in normotensive mice blood pressure lowering seems to have little impact on atherogenesis. However, in our study metoprolol-induced blood pressure reduction had no apparent effect on the reduction of plaque growth and the increase in stability, because responders and non-responders to metoprolol had equal plaque sizes and no association was observed between changes in heart rate or blood pressure and plaque size. Plotting vulnerability scores against hemodynamic changes did not reveal any clear cut association between hemodynamic changes and plaque vulnerability or signs of rupture either, suggesting that the hemodynamic effects of the double hit did not induce and that the hemodynamic effects of metoprolol did not prevent growth of unstable plaque. We concluded that plaque load or stability not associated with changes in hemodynamic
In order to assure that the effects were due to the β-adrenoceptor blocking effect and not to an unrelated effect of metoprolol, we treated a subgroup of animals with propranolol. The hemodynamic effects of propanolol treatment were comparably heterogenous and mild as for metoprolol. The effects on plaque size and complexity in propranolol treated animals were comparable to metoprolol treatment, suggesting that the observed effects are shared among different substances and substance classes of BRB. They may still be due to off-target pharmacology, but they are shared among more than one β-adrenoceptor blocker. We conclude that the anti-atherosclerotic effect of metoprolol in our study was not due to its heart rate and blood pressure lowering effects and that at least propranolol as another BRB had similar effects as metoprolol. We hypothesized that the effects on plaque size and vulnerability could be due to pleiotropic effects of metoprolol.

We therefore addressed the question if metoprolol could have affected vascular wall microenvironment. As noted above, extracellular matrix and MMPs are pivotal for the structure and composition of the vessel wall. It's well known that the MMP family degrades a wide spectrum of ECM proteins. MMP–associated ECM degradation is thought to contribute to the progression and rupture of atherosclerotic plaques.81 It has been reported that high levels of MMP-9 activity promote instability of plaques especially in advanced plaques.79 Human epidemiological and genetic studies have suggested MMP9 to be the strongest candidate inducing plaque rupture, with its expression correlating strongly to lesion instability and clinical manifestations of atherosclerosis.131-134 A recent
study has also revealed that MMP9 deficiency reduces the atherosclerotic lesion growth in Apo-E knockout mice.\textsuperscript{135} Furthermore, MMPs' inhibitor TIMPs has an effect of preventing plaque growth and promoting a more stable plaque phenotype.\textsuperscript{83,85} The balance of MMPs and TIMPs thus plays a crucial role in the process of collagen metabolism, which is important in stabilizing vulnerable plaques.\textsuperscript{136}

Remodeling phenomena in myocardium after infarction are governed by MMPs and their proteolytic activity. In addition has BRB after MI among other effects been shown to reorchestrate this proteolytic activity and specifically to induce TIMP2 expression.\textsuperscript{137,138} The equilibrium of MMP and TIMP activities in the vascular wall could, based on the observations in remodeling myocardium, be affected by metoprolol. We therefore addressed the question if in our animals TIMP-2-expression was altered by metoprolol. TIMP-2 expression increased significantly in the aorta of mice treated with metoprolol. We were also able to show that metoprolol reduced MMP9-associated gelatinase activity, which was likely due to the increase of TIMP-2 expression after metoprolol treatment since MMP9 expression at RNA level did not change. Thus metoprolol induced up-regulation of TIMP-2 expression in combination with the decrease of MMP9-associated gelatinolytic activity could improve plaque stability. This is in line with previous studies reporting that overexpression of TIMP-2 could inhibit atherosclerotic plaque growth and destabilization.\textsuperscript{85,139}

We reasoned that one particular cell type within the vascular wall could be responsible for the increased expression of TIMP2. However, in our experiments TIMP2 expression was not affected by metoprolol in human endothelial cells, VSMC or macrophages that had been stimulated with oxLDL and/or epinephrine.
Despite a very broad in vitro approach we were unable to model metoprolol induced TIMP2 expression in vascular wall cells. However, some authors report that in their hands BRB reduce inflammatory cytokines in human umbilical vein endothelial cells, VSMC and macrophages, which is interpreted as an anti-atherogenic effect.\textsuperscript{140-142} We therefore concluded that TIMP2 upregulation by metoprolol could be a complicated process that likely cell-cell or cell-matrix interactions.

Our study has numerous limitations. In our double hit model, the surgical procedure consists of only abdominal cavity opening with no further procedures. Also, the blood loss was not replaced by crystalloid volume, which only in part represents the situation in the operating room. The hemodynamic effects of metoprolol and propranolol were very mild. It is not possible to exclude that with stronger hemodynamic activation and stronger pharmacological effects our observations would have been different. An alternative method to decrease blood pressure could have been used to assure that it is not the hemodynamic effects that caused the prevention of plaque growth. Collagen content of the plaques was not measured, which could have shed more light on the question if proteolytic activity was indeed responsible for the effects that we observed. The animal model does address plaque rupture but not infarction or stroke. A clinically more relevant model or ultimately clinical trials would have to be done to ultimately address the question if plaque stabilization will also reduce clinically relevant endpoints.

Conclusion

In summary, our findings suggest that administration of metoprolol can reduce
atherosclerotic plaque growth in the stage of new plaque formation and enhance plaque stability both in the newly grown and advanced plaque. This effect is potentially mediated through upregulation of TIMP-2 expression in acute perioperative stress-dependent plaque growth and vulnerability. It also suggests that the long known effects of BRB on the precipitation of perioperative plaque rupture is likely not only due to modulation of heart rate and blood pressure but also by effects on the micro milieu in the vascular wall, among which proteolytic enzymes seem to play an important role. Based on our results it may be worthwhile to further investigate mechanisms that allow stabilization of plaques and prevent their growth with the ultimate goal to reduce their vulnerability and rupture in the perioperative phase.
5. Appendix

5.1 Reference:


122. Li JJ, Chen JL. Inflammation may be a bridge connecting hypertension and atherosclerosis. Medical hypotheses 2005;64:925-9.


5.2 Abbreviations

AHA  American Heart Association
αSMA  α-smooth muscle actin
AMI  Acute myocardial infarction
ApoE⁻/⁻  Apolipoprotein E-deficient
BCA  Bicinchoninic acid assay
BP  Blood pressure
BRB  β-adrenoceptor blockers
BSA  Bovine serum albumin
CD68  Cluster of Differentiation 68
cDNA  complementary Deoxyribonucleic acid
d  day
DAPI  4',6-diamidino-2-phenylindole
DMEM  Dulbecco’s modified Eagle’s medium
dNTP  deoxynucleotide triphosphates
ECM  Extracellular matrix
EGM  Endothelium Growth Medium
FITC  Fluorescein isothiocyanate
HBSS  Hank’s Balanced Salt Solution
H&E  Hematoxylin and Eosin
HPRT  Hypoxanthine-guanine phosphoribosyltransferase
HR  Heart rate
IEL  Internal elastic lamina
IgG  Immunoglobulin G
IHC  Immunohistochemistry
kg         kilogram
LDL      Low-Density Lipoprotein
LDLr−/−  LDL receptor−deficient
LV       Left ventricular
mg       milligram
MI       Myocardial Infarction
min      minute
ml       milliliter
MMP      Matrix metalloproteinase
µl       microliter
M.O.M    mouse on mouse
PBS      Phosphate Buffered Saline
PMI      Perioperative myocardial infarction
p.o.     Per oral
qRT-PCR  Quantitative real-time polymerase chain reaction
RNA      Ribonucleic acid
RPMI     Roswell park memorial institute
RT       Room temperature
sec      second
TIMP     Tissue inhibitor of metalloproteinase
Vol      Volume
VSMC     Vascular smooth muscle cell
5.3 Erklärung

Muster der Erklärung nach § 3 Abs. 2 Nr. 7-10

Ich erkläre, dass ich die der Fakultät für Medizin und Gesundheitswissenschaften zur Promotion eingereichte Dissertation mit dem Titel **Metoprolol increases TIMP2 expression in mice bearing acute complex atherosclerotic plaque** in der Abteilung für Experimentelle Anästhesiologie der MHH unter Betreuung von Prof. Dr. med. Gregor Theilmeier in Zusammenarbeit mit Houra Loghmani Khouzani ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden.

Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.


Ergebnisse der Dissertation wurden/werden in folgendem Publikationsorgan veröffentlicht: eventuell Shock

Falls zutreffend füge ich dem Promotionsgesuch eine ausführliche Erklärung bei, aus der hervorgeht, ob und wenn ja, welche Teile der Dissertation bereits in einer anderen wissenschaftlichen Arbeit, z. B. für studiengangbezogene Leistungen, verwendet wurden.

Ich erkläre, dass die Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg von mir befolgt wurden.

Oldenburg, den **12.08.2015**

_______________________________
(Unterschrift)
5.4 Zusammenfassung


Die vorliegende Studie zeigt, dass eine Behandlung mit Metoprolol die Plaquegröße bei Apo-E-Knockout-Äusen in der frühen Phase der Plaque-Progression reduzieren konnte, aber die Größe fortgeschrittener Plaque nicht. Jedoch gibt die für die Bewertung der Anfälligkeit von Plaques für Rupturen nach einem Morphologie-Score Anlass zu der Annahme, dass durch Metoprolol die Komplexität der Plaque sowohl in frühen als auch in späteren Stadien der Plaque-Entwicklung verringert wird. Die Reduktion des Plaquelastums und die Erhöhung der Stabilität waren jedoch weder auf die hämodynamischen Veränderungen durch Betablocker noch auf die Wahl der Substanz
zurückzuführen. Wir haben aber feststellen können, dass Metoprolol die Expression von TIMP-2 in dem untersuchten Gefäß hochregulieren konnte. Wie wir wissen, kann TIMP-2 das atherosklerotische Wachstum und die Destabilisierung von Plaques verhindern.

5.5 Curriculum vitae

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Participated in project of *Malignant arrhythmia after cardiopulmonary resuscitation* (supported by Natural Science Foundation of China, No. 30801082).

**Publication:**


**Abstracts:**


RGS5 is decreased in unstable human carotid plaque, and plays a role in the vascular smooth muscle cell/endothelial cell recruitment in vitro. H Loghmani khouzani, D Knoop, N Hu, F Echtermeyer, C Lankohr, C Tiemann, G Torsello, G Theilmeier. Poster presentation in the European Society of Cardiology (ESC) 2013, Amsterdam, Netherland
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